CHAPTER 3

STRESS RESPONSE

1. AEROBIC CELLS

Although certain terms, such as "heat shock", are widely used in the published literature on stress tolerance, many research laboratories generally adopt their own characteristic experimental protocols. These may appear on first sight to be similar to other laboratory protocols, however, subtle differences in experimental procedures in stress tolerance studies often have a marked effect on the observed results and, indeed, on interpretation of results. It is therefore necessary to define the terms adopted in experimental protocols on stress tolerance studies. In the present studies, the term "*heat shock*" refers to experiments in which cells cultured at 25°C are exposed to 37°C (a non-lethal temperature) for 45 min. The term "*heat stress*" refers to experiments in which cells cultured at 25°C are exposed either (a) directly to 48°C over a time course or (b) heat shocked at 37°C/45 min and then exposed to 48°C over a time course.

The ability of cells, previously subjected to a heat shock (37°C/45 min), to survive at 48°C is termed *induced thermotolerance*. On the other hand, the ability of cells to survive when exposed directly to a temperature shift from 25°C to 48°C is termed *intrinsic thermotolerance*.

It has recently been documented that stress tolerance of yeast is strongly influenced by growth rate and phase (Lewis *et al.*, 1993). For the present studies, all aerobic cells were grown on 2 % glucose YEP to mid-to-late exponential phase, corresponding to an absorption at 600 nm of 0.3-0.4 (approximately $1x10^7$ cells/ml) and a glucose concentration (residual) of about 0.5 % (w/v). Using the nomenclature of Lewis *et al.* (1993), this would correspond to the respiro-fermentative growth phase.

1.1. Thermotolerance

Numerous experiments were conducted to establish conditions under which significant differences in survival, as measured by colony forming units on an agar plate (Methods 2.5), of cells subjected to various heat stress protocols were noted.

The long established protocol of heat stressing yeast cells to 52°C for 5 min (McAlister and Finkelstein, 1980; Watson *et al.*, 1984) was abandoned in favour of measuring % survivors at 48°C over a 120 min time course. The former protocol, essentially a single point at a highly lethal temperature, does not allow for a clear distinction to be drawn between survival of cells which have been subjected to various heat treatments. On the other hand, the latter protocol (heat treatment at 48°C) involves the plotting of percentage cell survival over a relatively long time course and hence much clearer differences emerge in percentage survival of cells subjected to different heat treatments.

The marked differences in thermotolerance between heat shocked and heat stressed cells are shown in Fig. 3.1. For example, after exposure of cells to 48°C for 10 min, heat shocked cells exhibited >80% survivors in comparison to about 10% survivors for heat stressed cells. Similarly, after 60 min at 48°C, heat shocked cells exhibited >30% survivors in marked contrast to heat stressed cells (<0.1 %). It should be noted that very low % survivors are relatively easy to estimate, given the nature of the dilution plate count method.

1.2. Effect of glucose

Most experiments were conducted on cells which had been washed once with YEP media (minus glucose) before exposure to stress. This protocol was adopted in order to remove residual ethanol (0.2 - 0.4% w/v for mid-log phase cells; Lewis, personal communication) which had accumulated during the batch fermentation on 2% glucose. Furthermore, it was noted that unwashed cells subjected to stress in the growth media survived better, at

least initially, then cells washed free of growth media (Table 3.1). In this experiment, the thermoprotective effect of the growth media was evident up to about 60 min exposure to 48°C. However, after 120 min at 48°C, similar % survivors were noted in washed and unwashed cells (Table 3.1). For the sake of consistency, all experiments on stress tolerance, unless otherwise stated in the text, were conducted on cells washed free of growth media.

Table 3. 1. Percentage survivors following heat treatment in unwashed and washed cells. Cells were exposed to heat treatment either in the culture media (unwashed cells) or after one wash and resuspension in YEP-media minus glucose (washed cells). Results are the average of three separate experiments with standard errors in parenthesis.

TREATMENT	% SURVIVORS			
	Time (min) at 48°C			
	15	**30**	**60**	**120**
Washed cells	85 (±3)	67 (±2)	25 (±7)	2.6 (±2.1)
Unwashed cells	116 (±15)	93 (±0.7)	31 (±6)	2.2 (±1.1)

1.3. Oxidative and ethanol tolerances

In addition to heat, two other stresses, hydrogen peroxide (H_2O_2) and ethanol, were examined in the present studies. For comparative purpose, it was appropriate that suitable concentrations of these two stressors be selected in order to provide optimum percentage survivors comparable to that observed for the thermotolerance studies. Following preliminary experiments

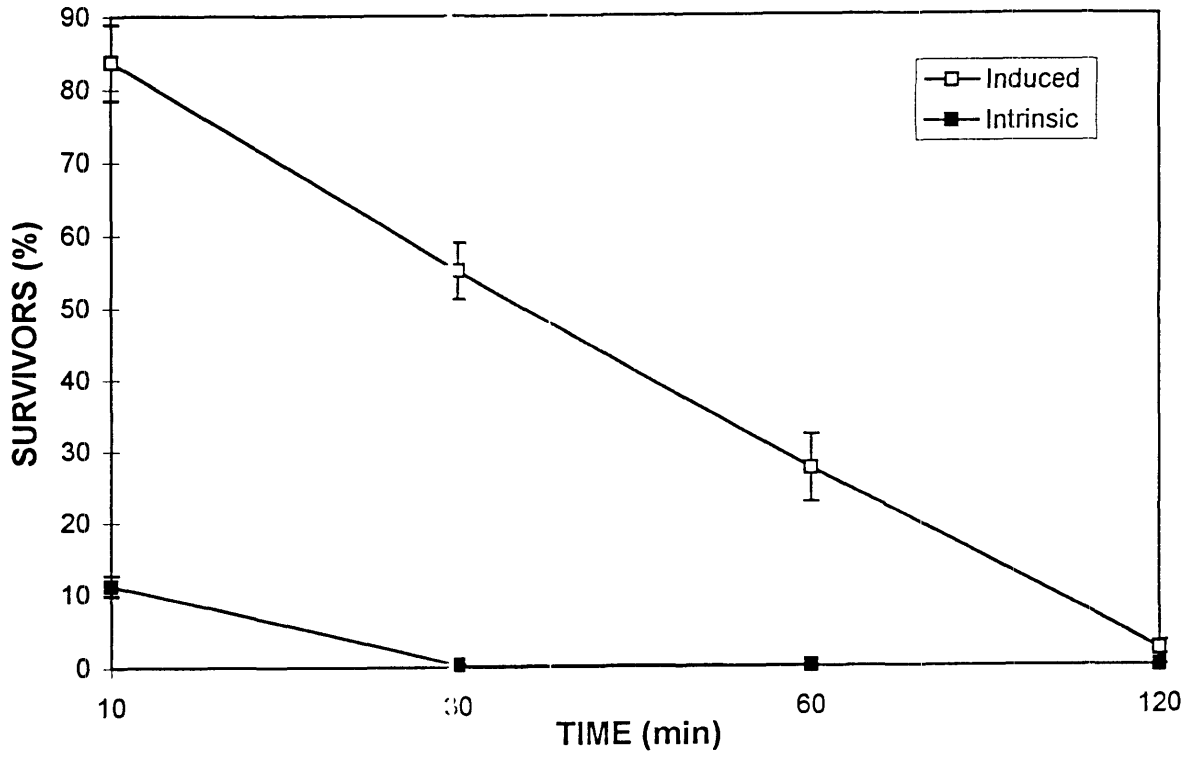

Fig. 3.1 Effect of heat treatment on cells grown aerobically at 25°C in YEP-media. Cells at mid-to-late logarithmic phase were washed in YEP-media and then heat stressed directly from 25°C to 48°C (intrinsic thermotolerance) or heat shocked from 25°C to 37°C for 45 min prior to heat stress at 48°C (induced thermotolerance). Induced thermotolerance (open symbol) and intrinsic thermotolerance (filled symbol) were expressed as survivors (%) compared to untreated control cells. The results represent the average of three separate experiments, with standart errors.

(results not shown), it was established that H_2O_2 concentrations of 5 mM and 10 mM and 16% (v/v) ethanol gave optimal percentage survivors over a 120 min incubation period (Fig. 3.2).

In the particular experiment illustrated in Fig. 3.2, a 15 min incubation of cells with 5 mM H_2O_2 actually resulted in > 100% survivors, relative to the control. This value decreased to just below 100% after 30 min, <40% after 60 min and finally <1% at 120 min. A higher concentration of H_2O_2 (10 mM) resulted in relatively rapid cell death from <80% survivors at 15 min to 20% by 30 min. Cell survival after 60 min and 120 min was <1%.

The kinetics of cell death in 16% ethanol was somewhat different to H_2O_2 induced death. Following an initial rapid drop to approximately 65% survivors after a 15 min incubation, cell survival was maintained at approximately 60 % and 45 % after incubation for 30 and 60 min respectively (Fig. 3.2). This was followed by a further rapid drop in survivors to 20% after 120 min in ethanol.

2. ANAEROBIC CELLS

For optimum growth of yeast in the absence of oxygen, the growth media has to be supplemented with a sterol and an unsaturated fatty acid as oxygen is required to synthesise these essential membrane components (Andreasen and Stier, 1953; 1954). In the present studies, ergosterol and unsaturated fatty acid, either oleic (C 18:1) or linolenic (C 18:3) acid were added to the anaerobic growth media. The addition of these fatty acids resulted in the enrichment of C 18:1 or C 18:3 into the yeast membranes (see section 2.1.4). The response to various stresses of anaerobically grown yeast with different membrane lipid compositions was examined in this section.

2.1 Thermotolerance

In this experiment, and for comparative purposes, the thermotolerance of aerobically grown cells was used as a control. As shown in Fig. 3.3, similar

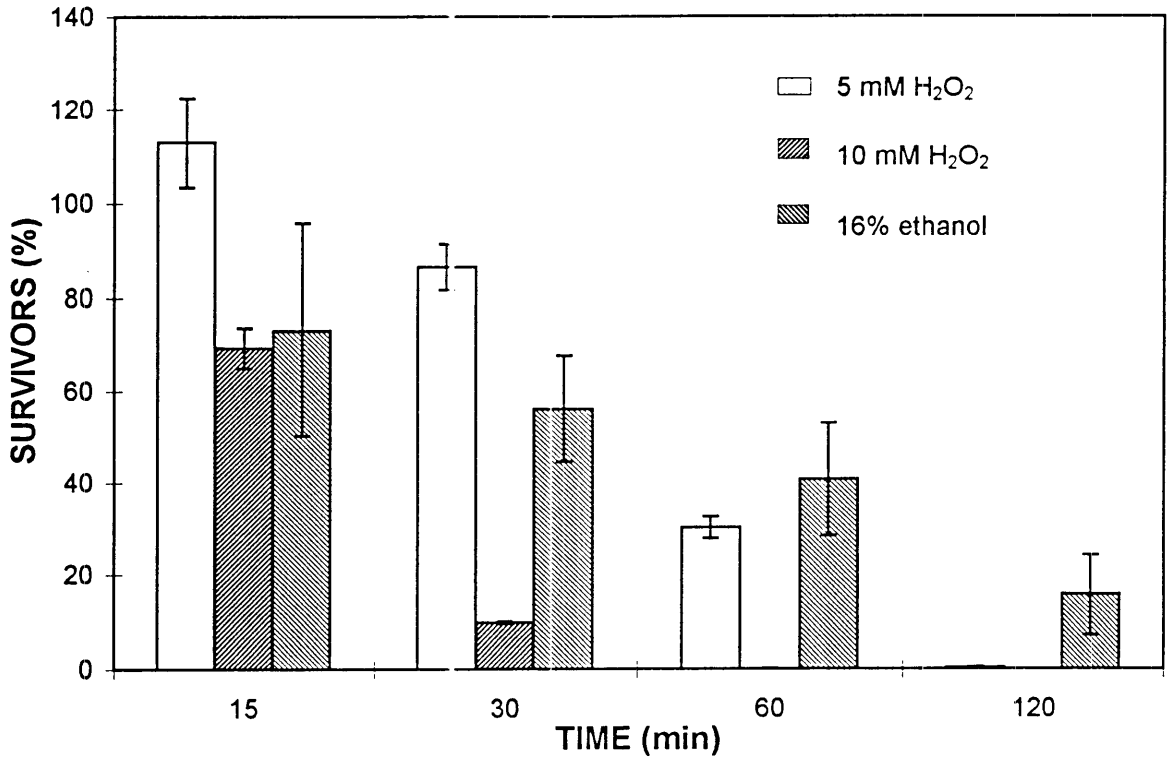

Fig. 3.2 Effect of H_2O_2 (5 mM or 10 mM) or ethanol (16% v/v) on cell survival in aerobically grown cells. Mid-to-late log phase cells were challenged with H_2O_2 or ethanol stress over a time course at 25°C. % Survivors were obtained by comparing treated cells to untreated control cells. The results represent the average of three separate experiments, with standard errors.

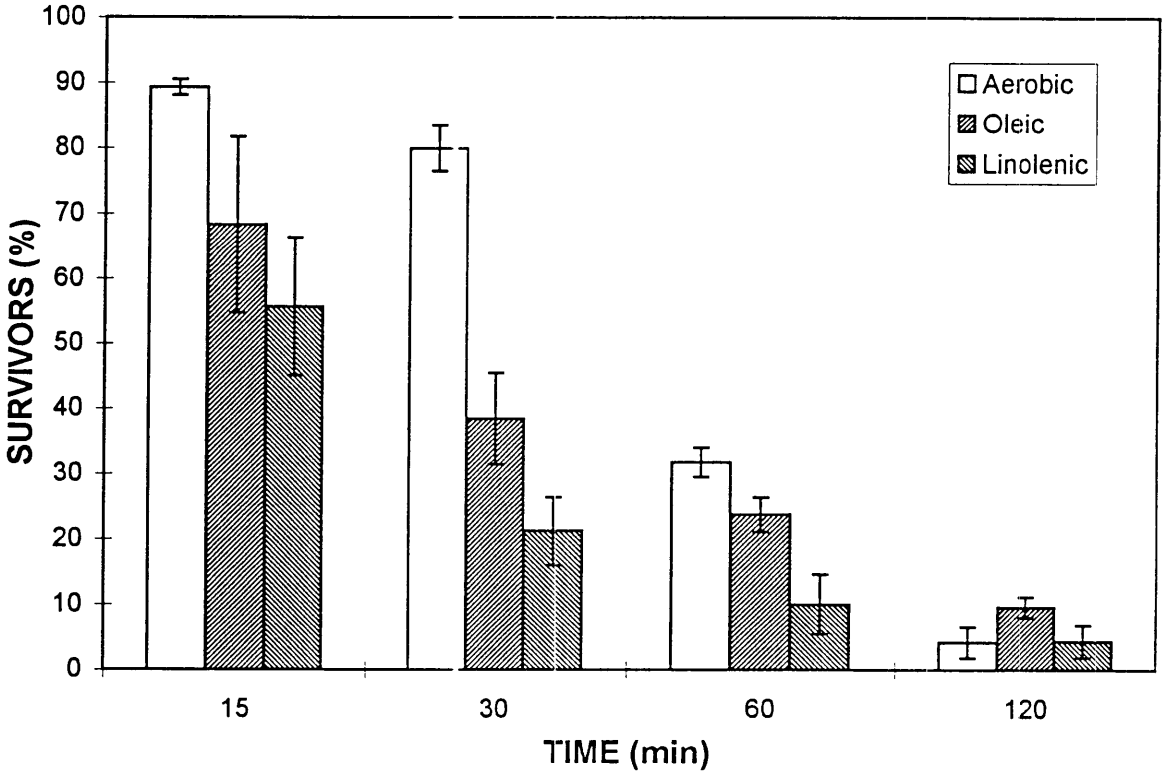

Fig. 3.3 Comparison of induced thermotolerance in aerobically grown cells and anaerobically grown cells supplemented with oleic (C18:1) or linolenic (C18:3) acids. Cells at mid-to-late exponential phase were challenged with a heat shock (37°C/45 min) followed by a heat stress (48°C) over a time course. The results represent the average of three separate experiments, with standard errors.

trends in response to thermotolerance was observed for cells grown aerobically or grown anaerobically with C18:1 or C18:3 as lipid supplements. Cells grown aerobically were the most thermotolerant with 90% survivors at 15 min, progressively decreasing to about 60 % and 40 % at 30 min and 60 min respectively. Anaerobic cells with membranes enriched in C18:1 showed a lower thermotolerance and cell survival progressively decreased from approximately 70% at 15 min to approximately 40% and to 25% after 30 and 60 min respectively. Anaerobic cells with membranes enriched in C18:3 were the most thermosensitive, with percentage survivors decreasing from about 60% at 15 min to about 25% and 10 % at 30 and 60 min respectively. A 120 min incubation at 48°C, resulted in a marked drop in % survivors for all three cell types. Here again, aerobic cells were the most thermotolerant (10% survivors) with the anaerobic cells at around 5% survivors. Exposure of the different cell types to a prior mild heat shock at 37°C for 45 min, resulted in a marked increase in thermotolerance to a 48°C heat stress. Although this observation was not unexpected, it was noteworthy that differences in induced thermotolerance of such heat shocked cells (i.e. aerobic versus lipid supplemented anaerobic cells) were significant (Fig. 3.3), in contrast to the intrinsic thermotolerances for aerobic cells and anaerobic cells (with oleic acid as supplement) as shown in Fig. 3.1 and 3.4, respectively.

2.2. Oxidative tolerance

Oxidative tolerance to H_2O_2 of cells grown anaerobically with C18:1 supplement is illustrated in Fig. 3.5. In a typical experiment, % survivors decreased from about 80% after 15 min exposure to H_2O_2 (5 mM) to about 60% at 30 min. This was followed by a rapid drop in cell survivors to about 10% and <1% after 60 min and 120 min respectively.

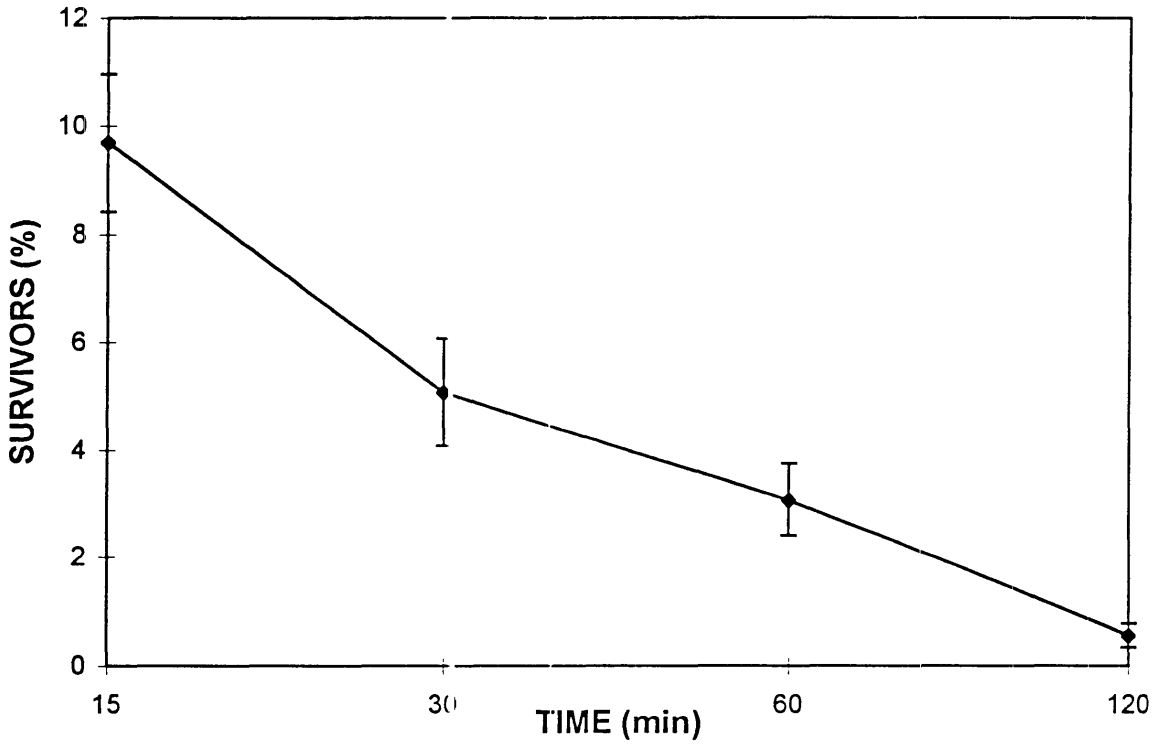

Fig. 3.4 Intrinsic thermotolerance of cells grown anaerobically with oleic acid as lipid supplement. Cells at mid-to-late exponential phase were heat stressed directly from 25°C to 48°C over a time course. The results represent the average of three different experiments, with standard errors.

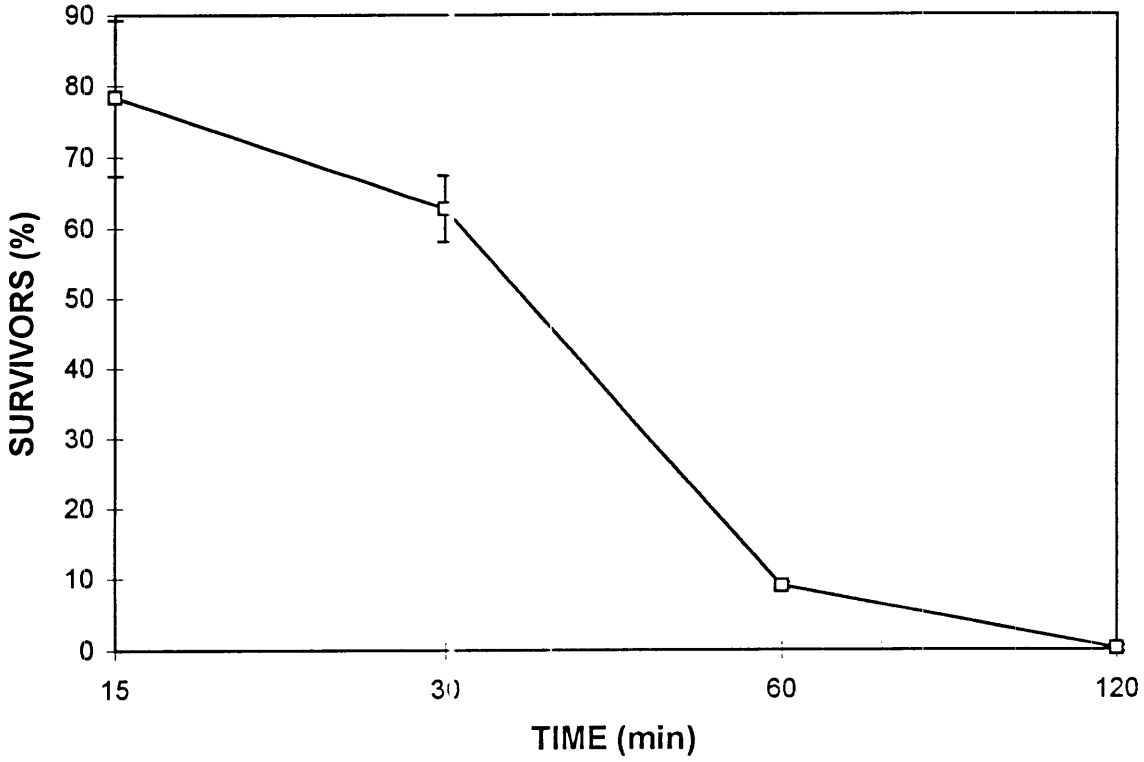

Fig. 3.5 Oxidative stress in cells grown anaerobically with oleic acid as lipid supplement. Cells at mid-to-late exponential phase were challenged to H_2O_2 (5 mM) at 25°C over a time course. Results presented are the average of three separate experiments, with standard errors.

3. ANTIOXIDANTS

The effect on thermotolerance of incubating cells with the naturally occurring antioxidants, glutathione and ascorbate, and the synthetic antioxidants, butylated hydroxytoluene (BHT) and propyl gallate (PG) was examined in this section.

3.1. Effect of adding antioxidant before treatment

3.1.1. Incubation with antioxidant before heat treatment

The antioxidants were added to growth media 15 min prior to exposure of cells to a heat shock/stress. Results are shown in Fig. 3.6. The addition of glutathione (10 mM) improved cell survival to some extent, compared to the control cells, at least at 15 min and 30 min heat shock/stress. However, after 60 min there was little difference in % survivors. At higher concentrations (20 mM) of glutathione, cell survival after 15 min at 48°C was markedly lower (approximately 60%) as compared to control cells (approximately 85%). There was little difference after 30 min, but differences were once again apparent after 60 min, with about 45% in controls (minus glutathione) and about 25% with glutathione. In general, the addition of ascorbate (10 mM or 20 mM) did not markedly affect % survivors on exposure of cells to a heat shock/stress.

The addition to growth media of the synthetic antioxidant BHT (100 or 200 ppm) prior to heat shock/stress, did not have a marked affect on cell survival. However, at much higher concentrations (750 ppm), cells were more thermosensitive in the presence than in the absence of BHT (Fig. 3.7).

Addition of propyl gallate (100 or 250 ppm), before heat treatment, had a marked effect on cell survival with < 1 % cells remaining viable in 2 hr compared with 13 % for control cells. A higher concentra-

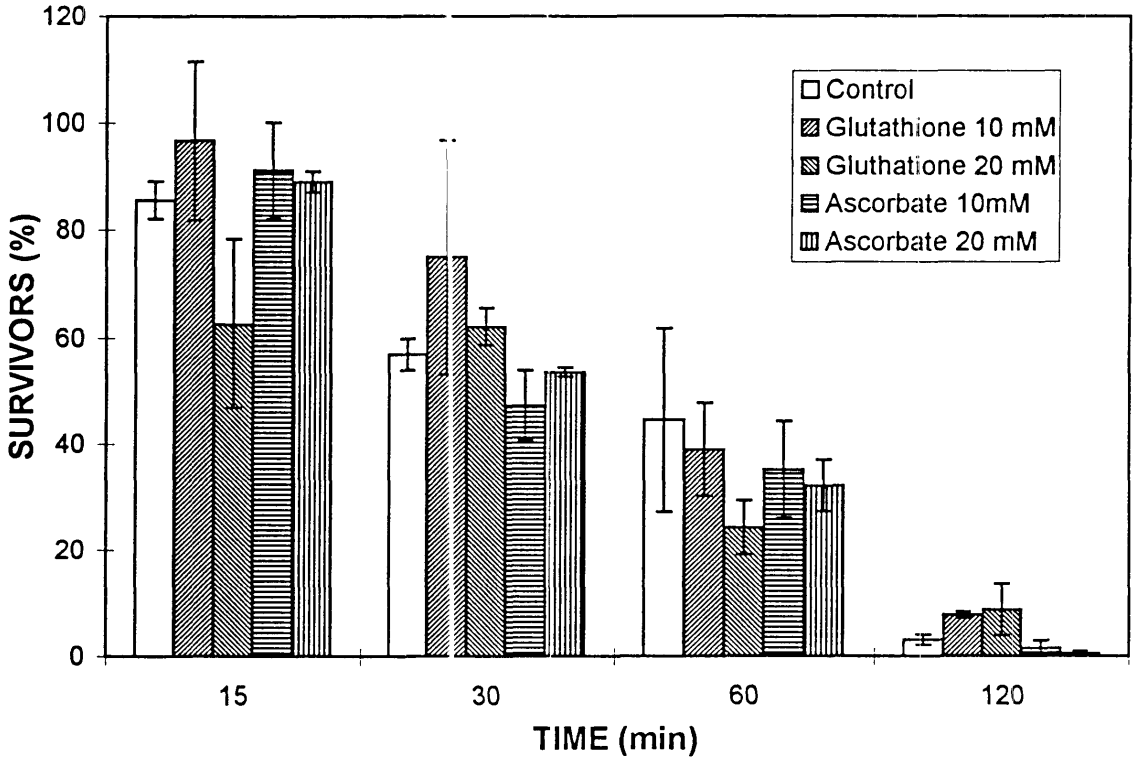

Fig. 3.6 Effect of antioxidants (glutathione and ascorbate) on induced thermotolerance. Cells grown aerobically at 25°C were preincubated with the antioxidants for 15 min prior to heat treatment. The latter was at 37°C for 45 min followed by 48°C over a time course. Results are the average of three separate experiments, with standard errors.

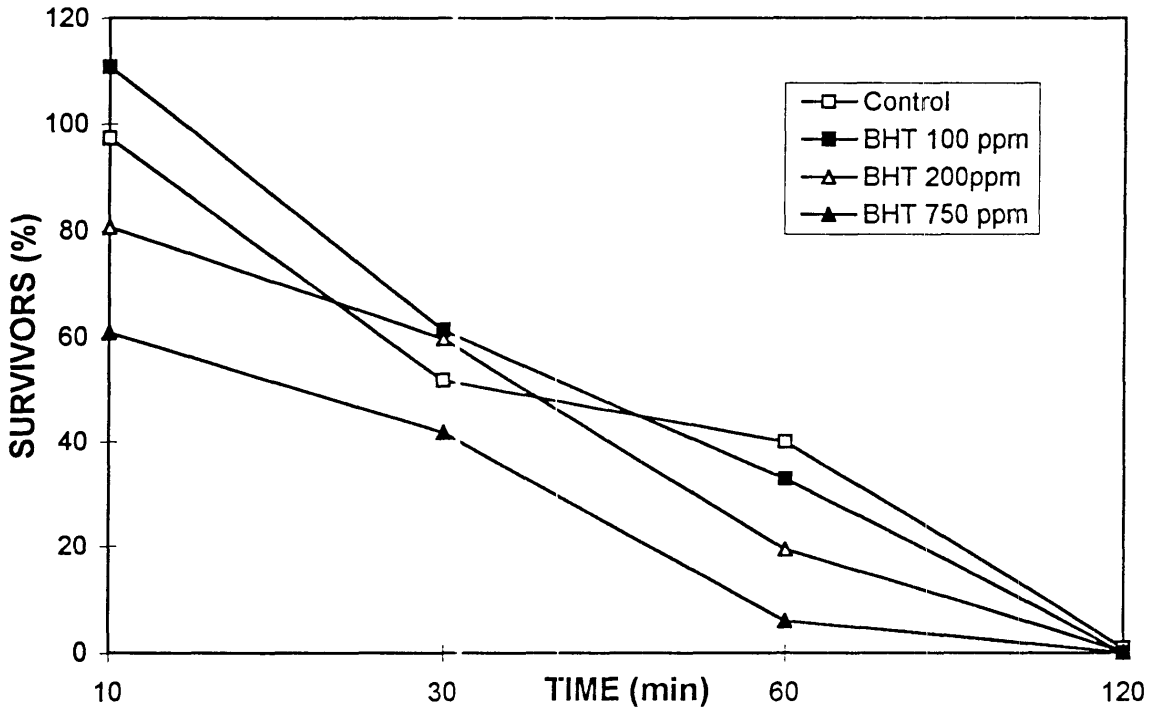

Fig 3.7 Effect of butylated hydroxytoluene (BHT) on induced thermotolerance of cells grown aerobically. Cells were preincubated (15 min) at 25°C with BHT prior to heat treatment. The latter was at 37°C for 45 min followed by 48°C over a time course. Results are from a typical experiment, and similar trends were observed in two other experiments.

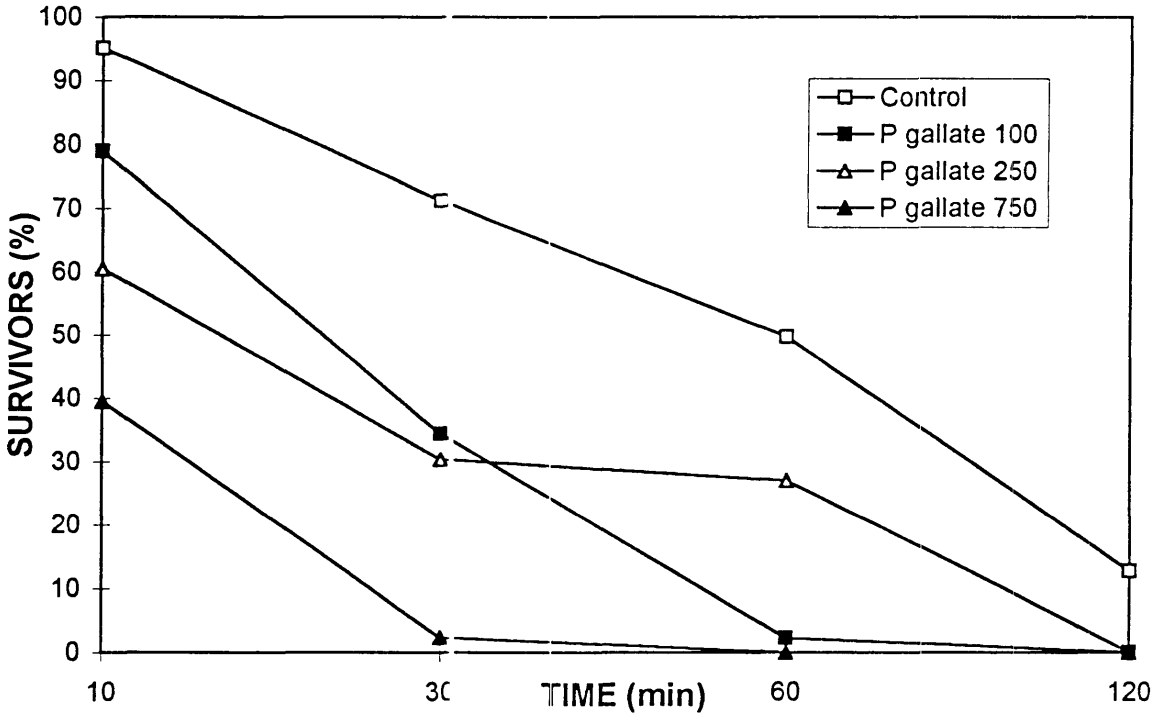

Fig. 3.8 Effect of propyl gallate (PG) on induced thermotolerance of cells grown aerobically. Different concentrations (100, 250 and & 750 ppm) were added. Cells were preincubated (15 min) at 25°C with PG prior to heat treatment. The latter was at 37°C for 45 min followed by 48°C over a time course. Result are from a typical experiment, and similar trends were found in two other experiments.

tion of propyl gallate (750 ppm) gave a near lethal effect in 60 min with less than 5% cell survival during a 30 min incubation and <1% during a 60 min incubation (Fig. 3.8).

3.1.2. Oxidative stress

Preincubation of cells with ascorbate and glutathione improved cell survival, to some extent, to an oxidative stress imposed by H_2O_2 as shown in Fig. 3.9 and 3.10.

The protective effect of the antioxidants was most evident after 60 min exposure to H_2O_2. However, by 120 min the % cell survivors was as low as the control (<1%).

3.2. Incorporation of antioxidant in the plate

The incorporation into YEP-agar plates of the naturally-occurring antioxidants, ascorbate, α–tocopherol, ergosterol and β–carotene, on cell recovery following a heat stress was studied in a number of experiments.

The addition of ascorbate or α–tocopherol to YEP-agar media as compared to YEP-agar minus antioxidants, resulted in around a 5-10% increase in cell survival (Fig. 3.11 and 3.12). The protective effect of α–tocopherol was still evident in cells which had been exposed to a heat stress at 48°C for 120 min. On the other hand, under the latter heat shock/stress condition, there was essentially no difference in cell survival in the case of YEP-media plus ascorbate.

The addition of the antioxidants ergosterol or β–carotene to YEP-media had, in general, a detrimental effect on cell recovery and survival. As shown in Fig. 3.13 and Fig. 3.14 there was around a 5-10% drop in cell viability when , following a heat shock/stress, cells were plated out onto YEP-media plus ergosterol or β–carotene as compared to YEP-media minus antioxidant.

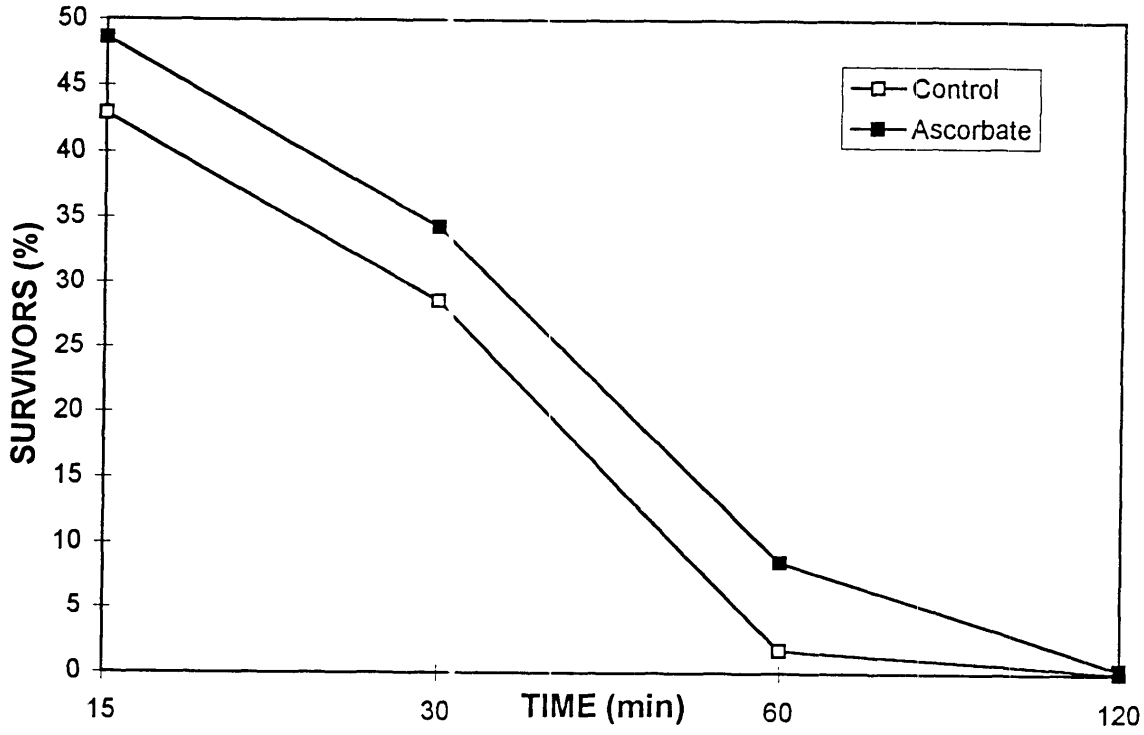

Fig. 3.9 Effect of ascorbate on oxidative stress in cells grown aerobically. Cells were preincubated (15 min) at 25°C with ascorbate (10 mM) prior to a H_2O_2 stress (10mM). Results from a typical experiment.

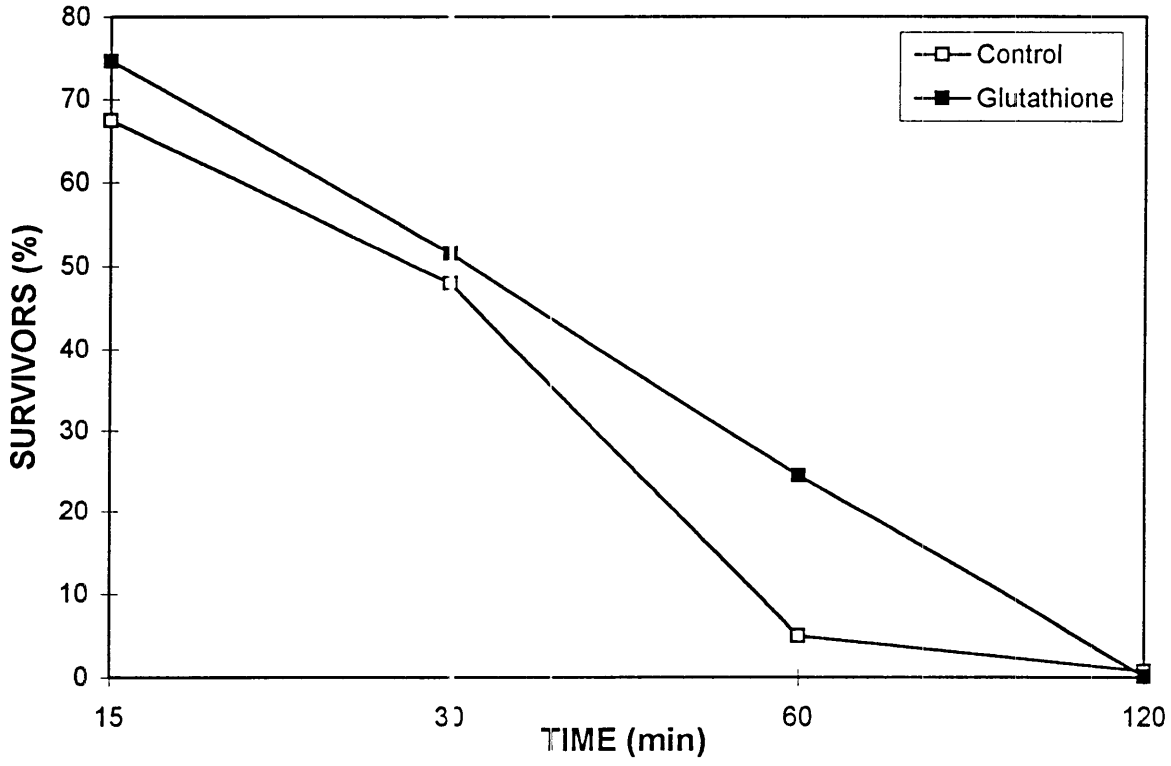

Fig. 3.10 Effect of glutathione on oxidative stress of aerobic cells. Cells were preincubated with glutathione (10 mM) for 15 min at 25°C prior to H_2O_2 challenge (10 mM) over a time course. Results from a typical experiment.

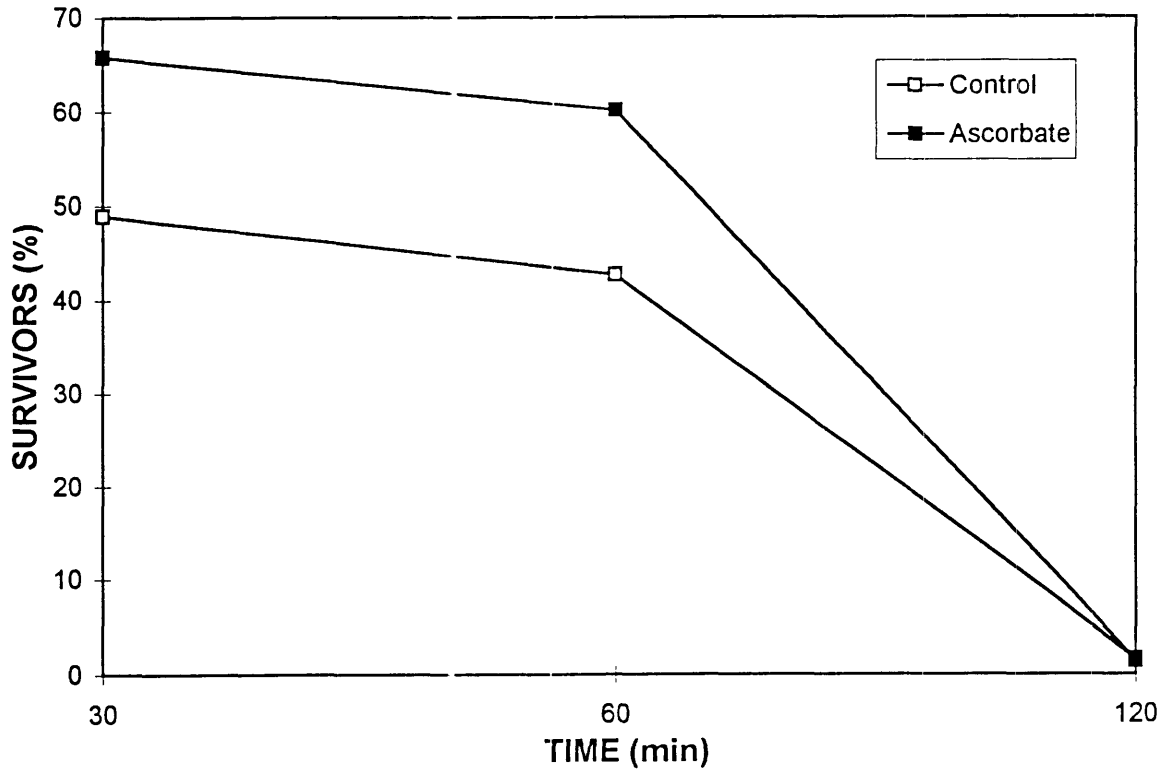

Fig. 3.11 Effect on induced thermotolerance of incorporation of ascorbate in YEP-agar media. Mid-log phase cells grown aerobically at 25°C were heat shocked (37°C/45 min) prior to heat stress (48°C) over a time course. Cells were then plated out on 2% glucose YEP-agar, with or without ascorbate (10 mM). Results from a typical experiment.

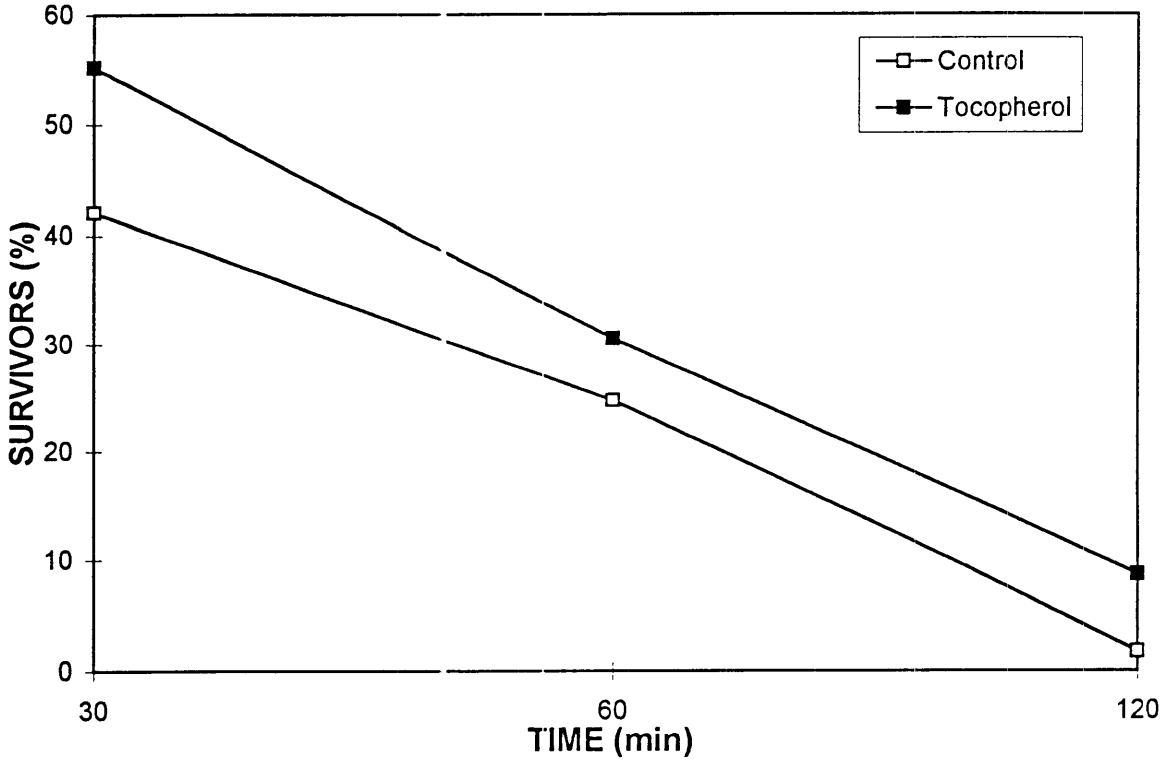

Fig. 3.12 Effect on induced thermotolerance of incorporation of α–tocopherol in YEP-agar media. Mid-log phase cells grown aerobically at 25°C were heat shocked (37°C/45 min) prior to heat stress (48°C) over a time course. Cells were then plated out on 2% glucose YEP-agar, with or without α-tocopherol (10 μM). Result from a typical experiment.

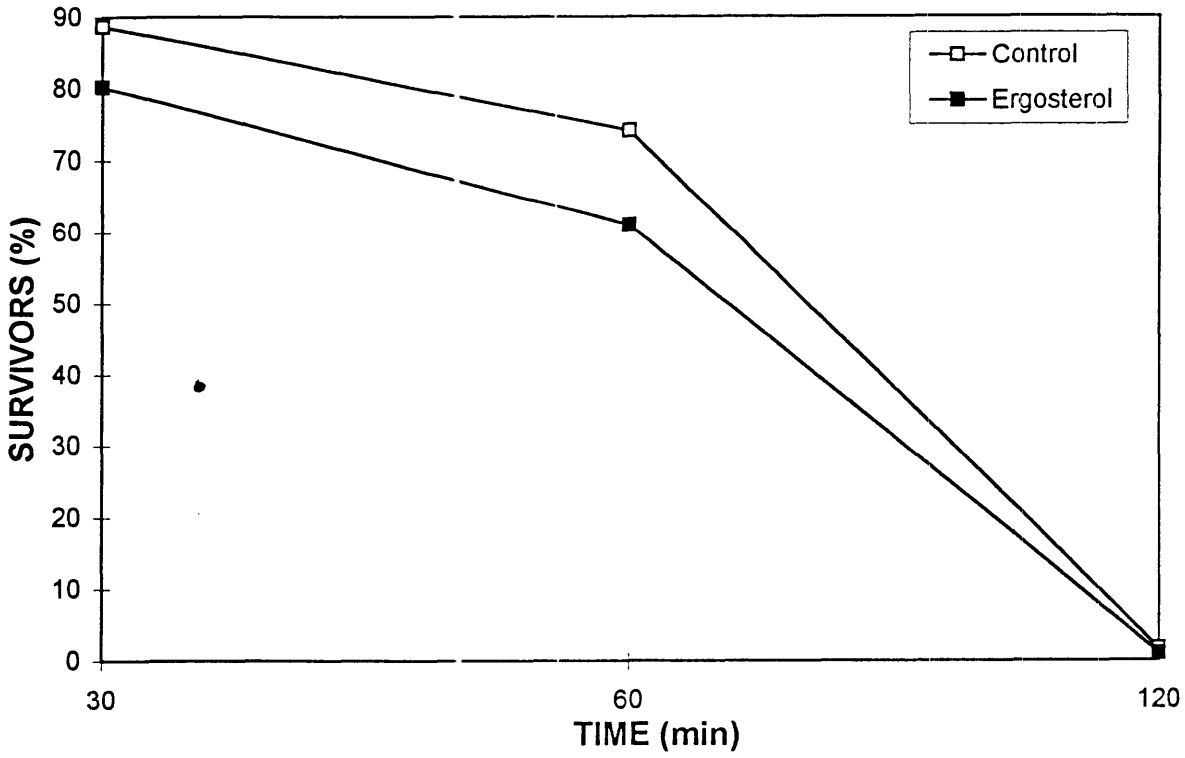

Fig. 3.13 Effect on induced thermotolerance of incorporation of ergosterol in YEP-agar media. Experimental detail as in previous figure legend except ergosterol (5 µg/ml) was incorporated into 2% glucose YEP-agar media. Result from a representative experiment.

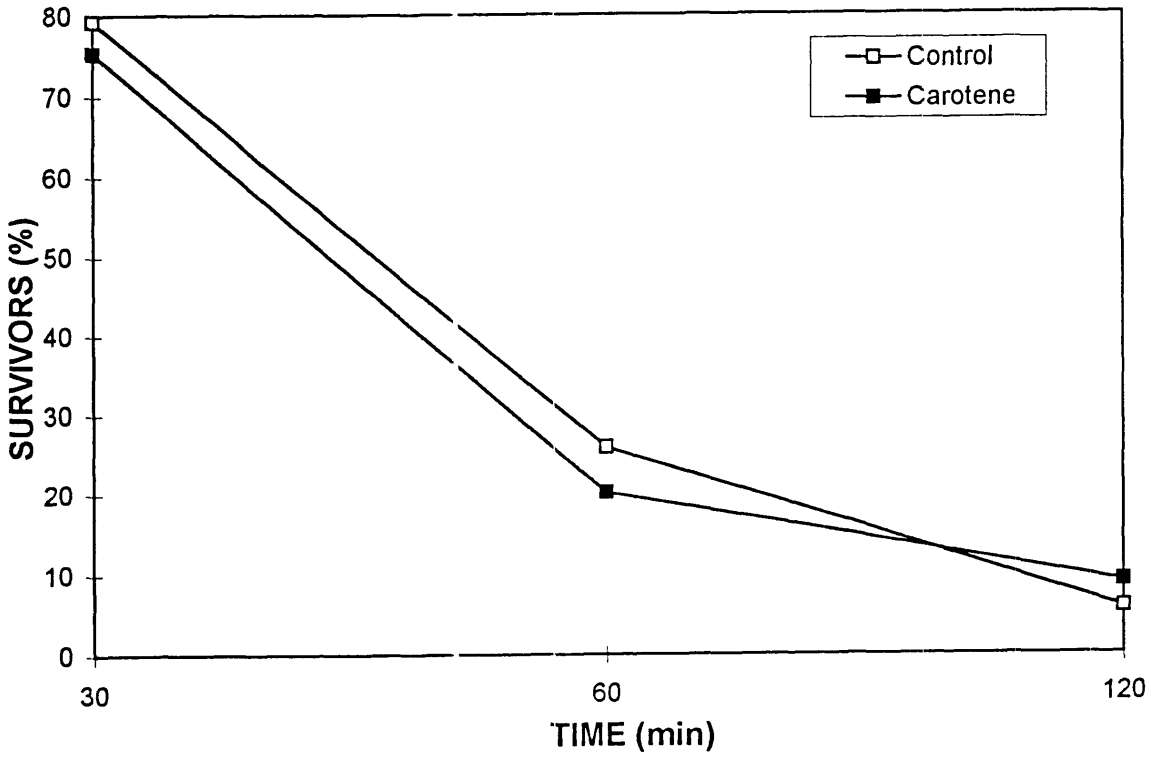

Fig. 3.14. Effect on induced thermotolerance of β-carotene in YEP-agar media. Experimental details as in Fig. 3.12 except β-carotene (10μM) was incorporated into 2% glucose YEP-agar media. Result from a representative experiment.

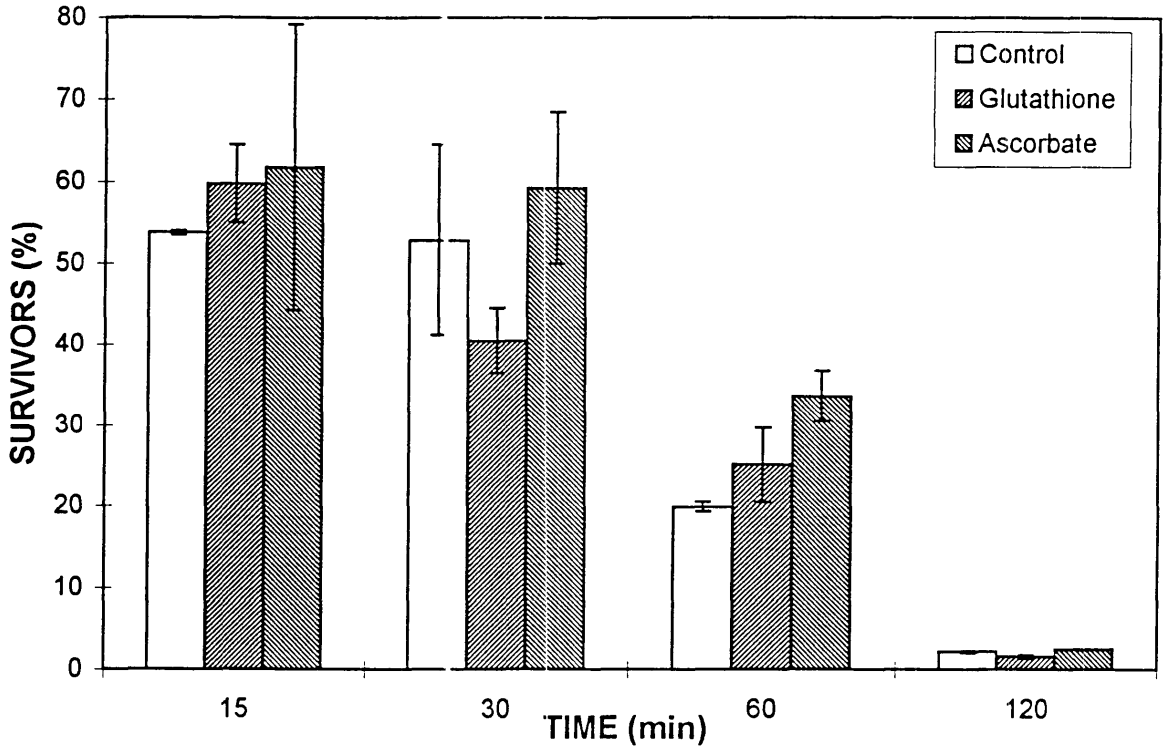

Fig. 3.15. Effect on induced thermotolerance of cells grown aerobically in YEP media with addition of antioxidant. Cells were grown aerobically at 25°C in 2% glucose YEP media with or without (control) antioxidant. Antioxidants were either glutathione (10 mM) or ascorbate (10 mM). Induced thermotolerance was determined as in Methods. Results represent the average of three separate experiments.

3.3. Incorporation of antioxidant in growth media

In this series of experiments, the effects on thermotolerance of incorporation into growth media of the antioxidants, glutathione and ascorbate were examined. The results are presented in Fig. 3.15.

Overall, the results indicated that cells grown in the presence of antioxidant, in particular ascorbate, were more thermotolerant (5-10%) than cells grown in the absence of antioxidant. However, there was no difference in cell survival (about 2%) following exposure of cells to a heat stress at 48°C for 120 min.

It should be noted that there was no difference in the growth characteristics over 48 hr between cells grown in the presence or absence of antioxidants (result not shown).

3.4. Effect of ascorbate on recovery of cells from heat stress

In this series of experiments, the effect of ascorbate on recovery of cells from heat stress was investigated. Following a mild heat shock (37°C for 45 min), cells were heat stressed at 48°C for 60 or 120 min. The heat stressed cells were then allowed a recovery period (5 hr or 10 hr) at 25°C either in YEP-medium or in 2% glucose-YEP-medium, with or without ascorbate (10mM).

Results indicated that there was essentially no difference in % survivors between cells allowed to recover (5 hr or 10 hr at 25°C) from heat stress in YEP-medium with or without ascorbate (Table 3.2). Interestingly, however, if cells were allowed to recover in 2% glucose-YEP-medium, there was a substantial increase in cell numbers. This was particularly evident in the experiment in which cells were heat stressed at 48°C for 60 min, and then allowed to recover for 5 or 10 hr at 25°C in 2% glucose-YEP-media, with or without ascorbate (Table 3.2, lines C and D, column 3 and 5). It was evident

from the results, that the cells which had survived the initial heat stress (columns 1 and 2) were capable of growth and division in 2% glucose-YEP, resulting in apparently >100% survivors (lines C and D, columns 3 and 5). Lower % survivors after 120 min at 48°C (column 2) was also reflected in lower cell number for cell recovery at 25°C after 5 hr (column 4) and 10 hr (column 6).

Table 3. 2. Effect of ascorbate during recovery from induced thermotolerance, in the presence or absence of glucose. In lane 1, the term 0/60 min refers to recovery time at 25°C (0 hr) and time at 48°C (60 min). Similarly for lanes 2 to 6. Results are the average of two separate experiments.

		% SURVIVORS					
	TREATMENT	*1*	*2*	*3*	*4*	*5*	*6*
		0/60 min	*0/120 min*	*5/60 min*	*5/120 min*	*10/60 min*	*10/120 min*
A	**Control**	18 (±5)	1.2 (± 0.01)	23 (± 7)	1.3 (± 0.03)	26.6 (± 1)	1.6 (± 0.08)
B	**Ascorbate**	14 (± 1.4)	1.3 (± 0.2)	25 (± 3.4)	1 (±0.75)	28 (± 9)	1.8 (± 0.08)
C	**Glucose**	19 (± 3.2)	1.3 (± 0.05)	93 (± 1.8)	7.3 (± 2)	3921 (± 1742)	10.5 (± 6.3)
D	**Glucose + ascorbate**	16.5 (± 1.6)	1.2 (± 0.04)	191 (± 23)	5.1 (± 2.2)	6747 (± 533)	18.6 (± 1.1)

CHAPTER 4

EFFECT OF OXYGEN

1. AEROBIC CELLS

1.1. Oxygen versus nitrogen

Two experimental protocols were adopted in this series of experiments. The first protocol involved continuous aeration of cultures with high quality oxygen or nitrogen during heat shock/stress (37°C for 45 min and continued to 48°C for 15, 30, 60 and 120 min). The second protocol involved continuous aeration of cultures with oxygen or nitrogen after the heat shock/stress treatments. The results of these experiments are summarised in Table 4.1. For comparative purposes, all results were normalised with respect to controls (expressed as cfu/ml) which were incubated for equivalent times, but not subject to oxygen or nitrogen aeration, at the same temperatures for induced thermotolerance or at 25°C for recovery.

It was observed that aeration with nitrogen during heat shock/stress treatments was detrimental to cell survival (<1 at all times). By contrast, aeration with oxygen was extremely beneficial to cell survival. There was a 10 to 15-fold increase, relative to normalised controls, in survival with cells which had been heat stressed (up to 60 min) in the presence of oxygen, and a 3-fold increase after 120 min (Table 4.1). It was also noteworthy that aeration with nitrogen (3-fold) or oxygen (approximately 10 to 15-fold), immediately after heat shock/heat stress, was beneficial to cell survival. This was especially true for oxygen aeration which, in some experiments, exhibited a 15-fold increase in cell survival (Fig. 4.1). On the other hand, oxygen aeration during heat stress (48°C) only was highly detrimental to cell survival (Fig. 4.2).

1.2. Oxygen aeration and heat, H_2O_2 or ethanol stress

In this series of experiments, the effect of oxygen aeration during thermal, oxidative or ethanol stress was compared. Results are summarised in Fig. 4.1 (heat), 4.3 (H_2O_2), and 4.4 (ethanol).

As previously indicated (Table 4.1) and shown in Fig.4.1, oxygen aeration during thermal stress had a marked beneficial effect on cell survival to a heat stress. Cell survival was high at 48°C up to 60 min, after which time (120 min) cell survival dropped sharply, although still higher than in control cells. Similar trends were observed in cells exposed to oxidative stress (Fig. 4.3, 5mM H_2O_2) or ethanol stress (Fig. 4.4, 16% v/v). In the case of ethanol stress, there was a progressive drop in cell survivors with time of exposure to ethanol and, in the case of oxidative stress, there was a marked drop in cell survivors following a 15 min to 30 min exposure to H_2O_2 (Fig. 4.3).

Table 4.1 Comparison between aeration with oxygen or nitrogen on induced thermotolerance. All results were normalised with respect to appropriate controls which were incubated at 48°C or 25°C without oxygen or nitrogen aeration. In the first series of experiments, cells at 25°C were heat shocked (37°C/45 min) and then heat stressed (48°C for 15, 30, 60 and 120 min), with either oxygen or nitrogen aeration throughout the heat treatments. In the second series of experiments, cells were heat shocked and heat stressed (48°C/60 min) before aeration at 25°C with oxygen or nitrogen for 30 min or 60 min.

	Time at 48°C				*Time at 25°C*	
	15	**30**	**60**	**120**	**30**	**60**
Oxygen	15	15	11	3	11	51
Nitrogen	0.7	0.4	0.35	0.2	3	3

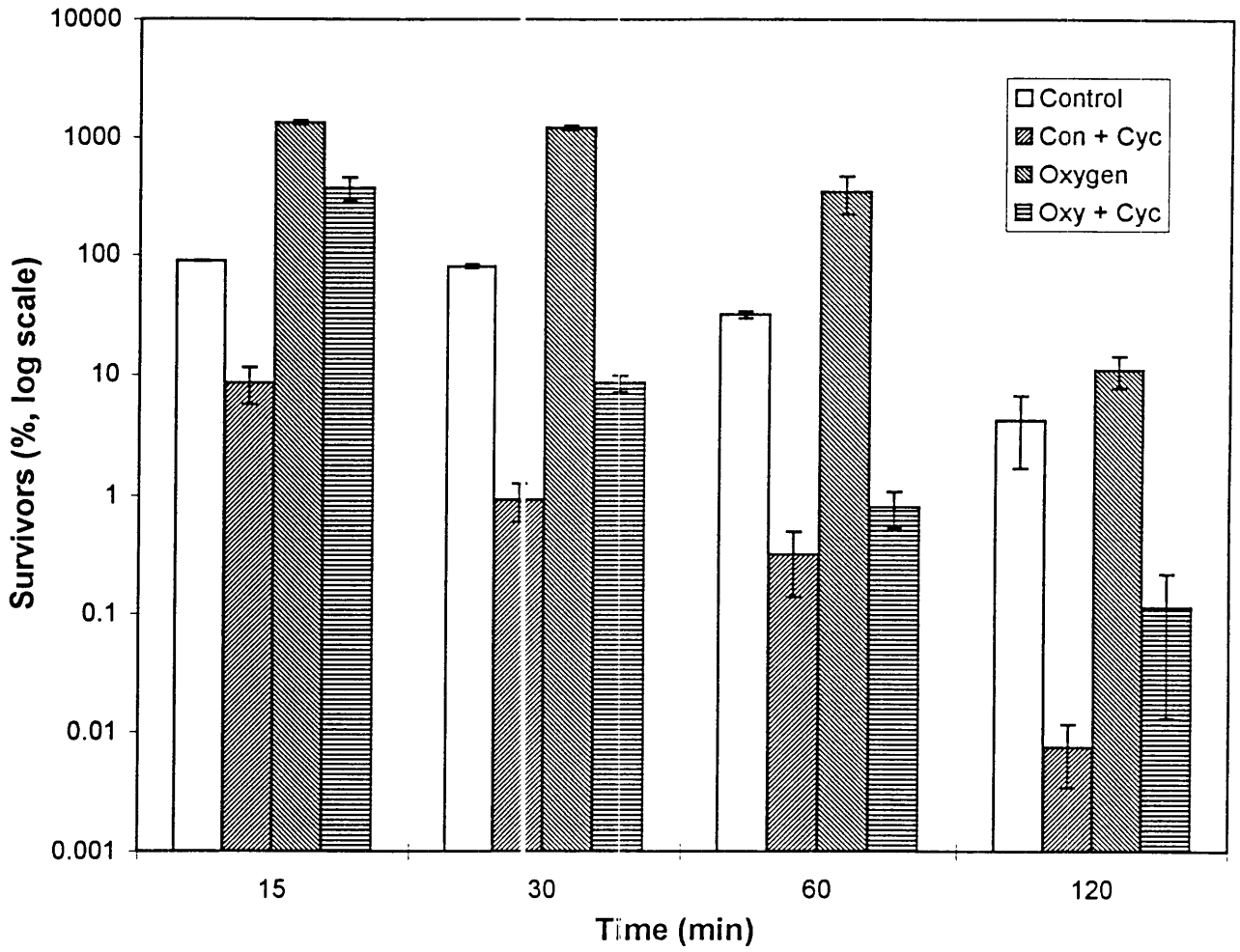

Fig. 4.1 Effect of oxygenation on induced thermotolerance, in the presence or absence of cycloheximide. Mid-to-late exponential phase cells were preincubated (15 min) with or without cycloheximide (50 μg/ml) before heat treatment. Cells were then heat shocked (37°C/45 min) and heat stressed (48°C) over a time course, with or without oxygenation. Results represent the average of three separate experiments, with standard errors.

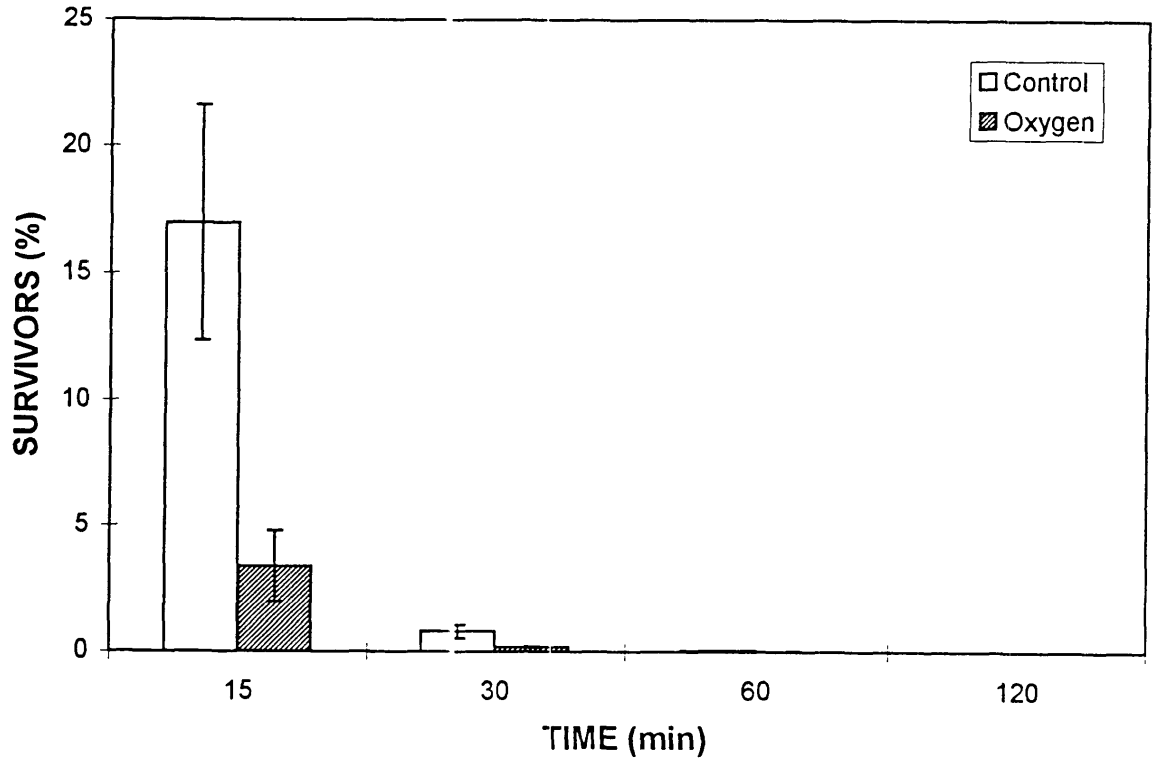

Fig. 4.2 Effect of oxygenation on intrinsic thermotolerance. Cells were grown aerobically at 25°C and then heat stressed directly with or without oxygenation, to 48°C over a time course. Results represent the average of three separate experiments, with standard errors.

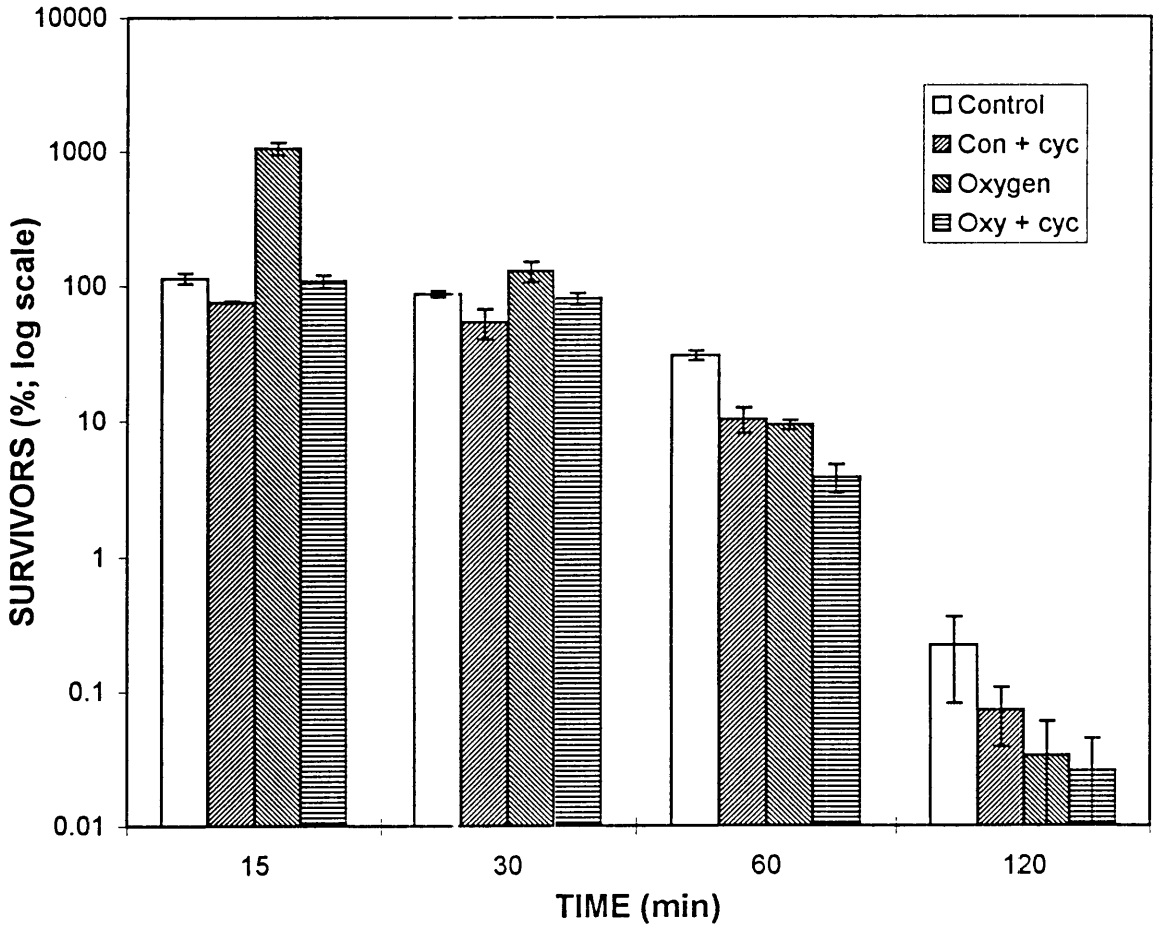

Fig. 4.3 Effect of oxygenation on oxidative stress. Cells were preincubated (15 min) with or without cycloheximide (50 μg/ml) prior to oxidative stress. For oxidative stress, cells were challenged with H_2O_2 (5mM) at 25°C over a time course, with or without oxygenation. Results shown are the average of three separate experiments.

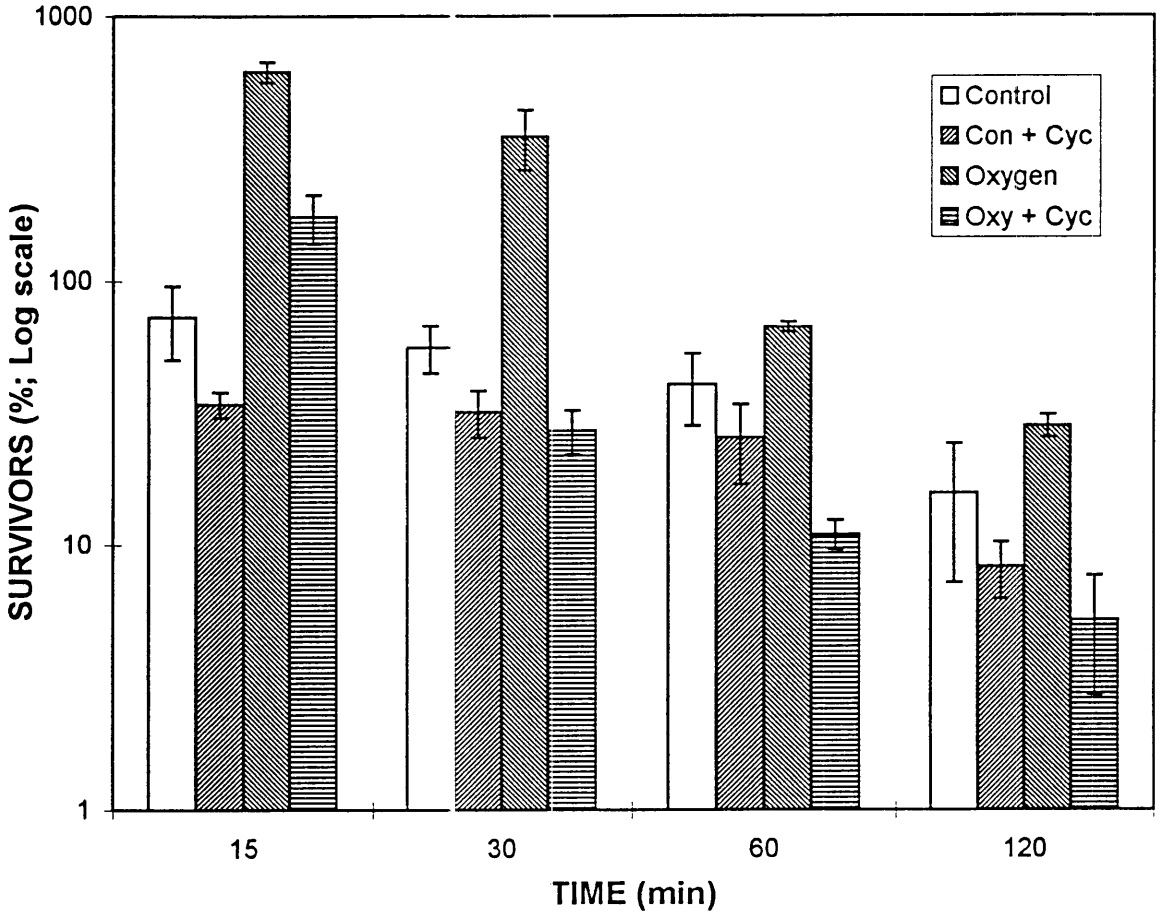

Fig. 4.4 Effect of oxygenation on ethanol stress. Cells were preincubated (15 min) with or without cycloheximide (50 μg/ml) prior to ethanol stress. For ethanol stress, cells were challenged with ethanol (16% v/v) at 25°C over a time course, with or without oxygenation. Results shown are the average of three separate experiments.

1.3. Effect of cycloheximide or thermotolerance and oxygen aeration

The effect of inhibiting protein synthesis with cycloheximide (50 μg/ml) on the oxygen aeration experiments, was examined. In these experiments, cells were preincubated for 15 min with cycloheximide prior to a heat shock/ heat stress and oxygen aeration. As illustrated in Fig. 4.1 for thermal stress, there was limited protective effect of oxygenation, when protein synthesis was inhibited with cycloheximide. The protective effect was only evident up to a 15 min heat stress at 48°C. Similar trends were noted for oxidative stress (Fig. 4.3) and ethanol stress (Fig. 4.4). Longer heat stress treatments inevitably lead to a decrease in cell survival for all three stresses.

1.4. Oxygen aeration prior to a heat shock/stress

Aeration with oxygen (60 min) prior to heat shock/stress lead to thermosensitisation of cells. As shown in Fig. 4.5, prior aeration for 1 hr with oxygen rendered cells significantly more thermosensitive to a heat stress. The increased heat sensitivity as compared to non-oxygenated controls, was particularly evident following exposure of cells to a heat stress for 15 min and 30 min (Fig. 4.5). Similar results were obtained in the case of cells heat stressed directly from 25°C to 48°C except, as anticipated, cell survival was lower for both control and test cultures (Fig. 4.6).

1.5. Oxygen aeration during recovery

Data from experiments designed to determine the effects of oxygen during recovery (after heat shock/stress, 45 min at 37°C and 30 min at 48°C and after stress, 30 min at 48°C) indicated that there was a substantial increase in cell survival and growth in the presence of oxygen (Fig. 4.7). On the other hand, there was little effect of oxygenation on cell survival following a heat stress (Fig. 4.8).

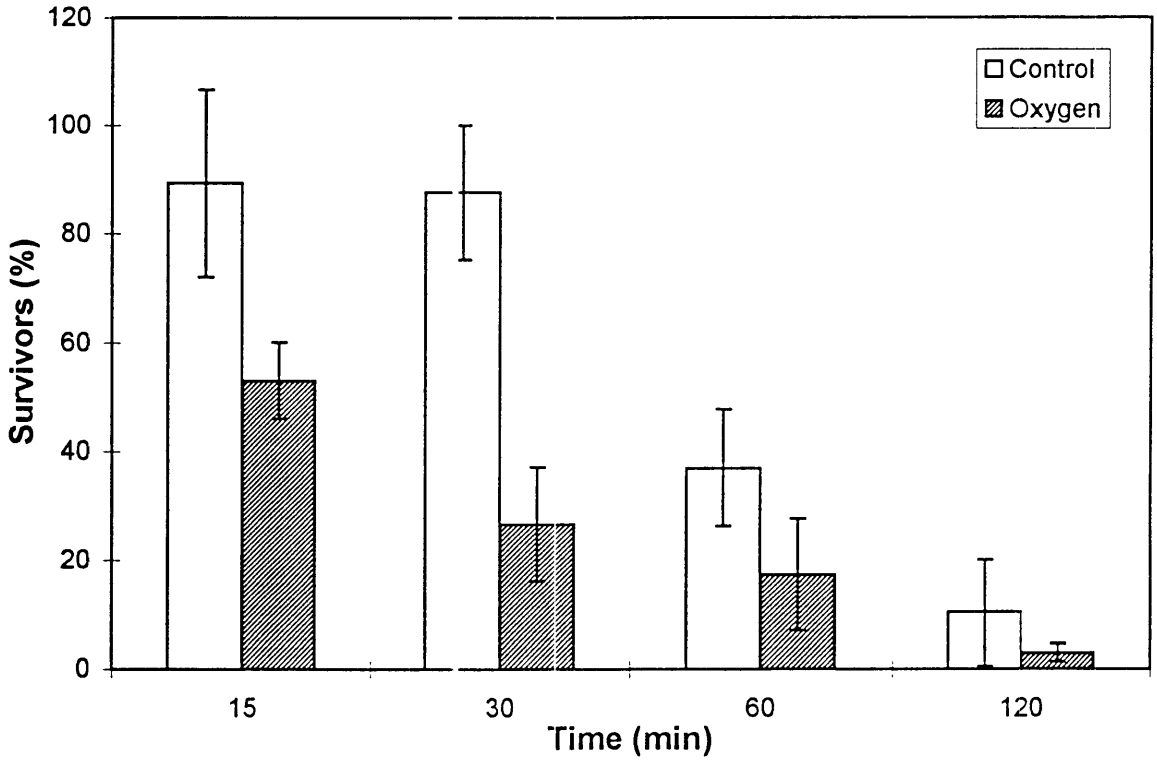

Fig. 4.5 Effect of a prior oxygenation on induced thermotolerance. Mid-to-late exponential phase cells were oxygenated (60 min) at 25°C by passing a continuous stream of high purity oxygen through the cultures. Cells were then heat shocked and then heat stressed over a time course. Results represent the average of three separate experiments.

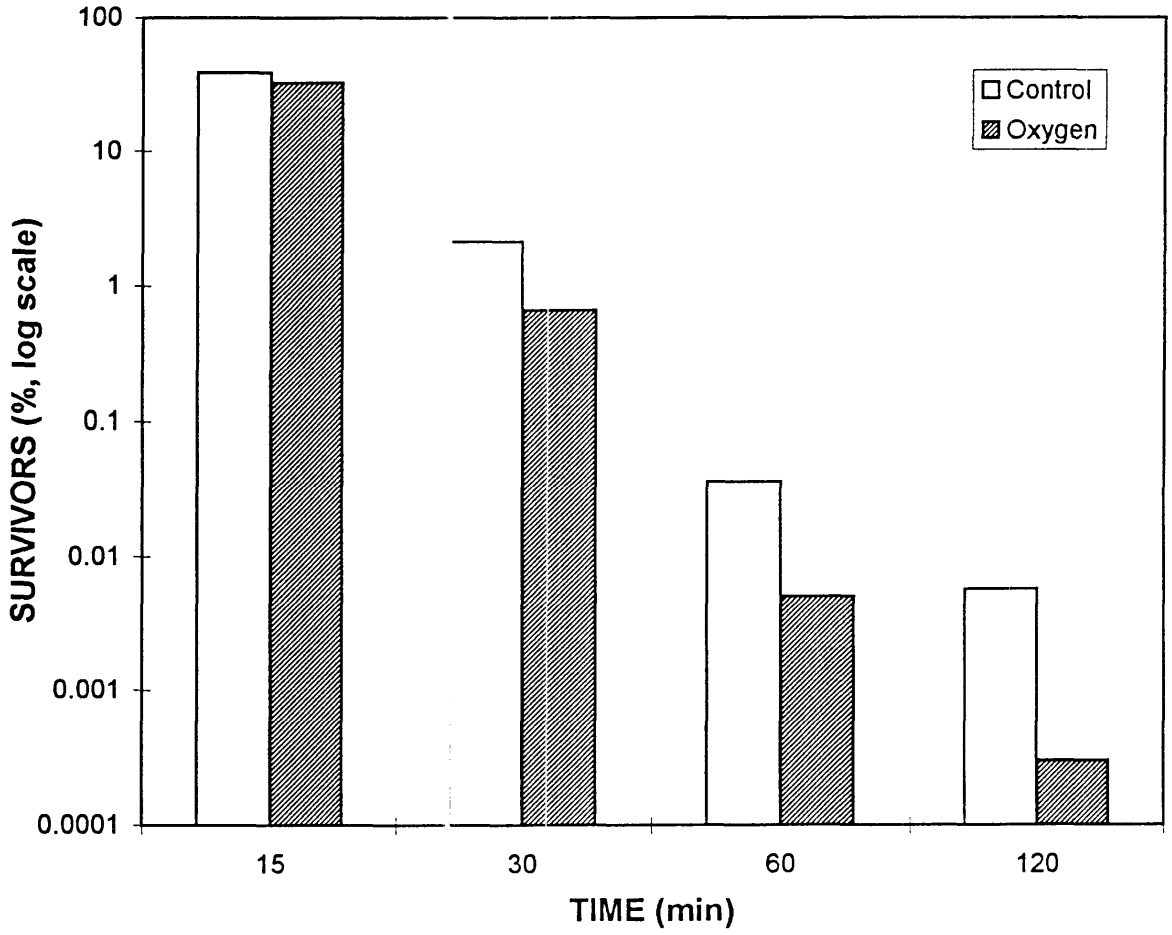

Fig. 4.6 Effect of prior oxygeration on intrinsic thermotolerance. Cells were oxygenated (60 min) with high purity oxygen prior to a heat stress (48°C) over a time course. Result from a typical experiment.

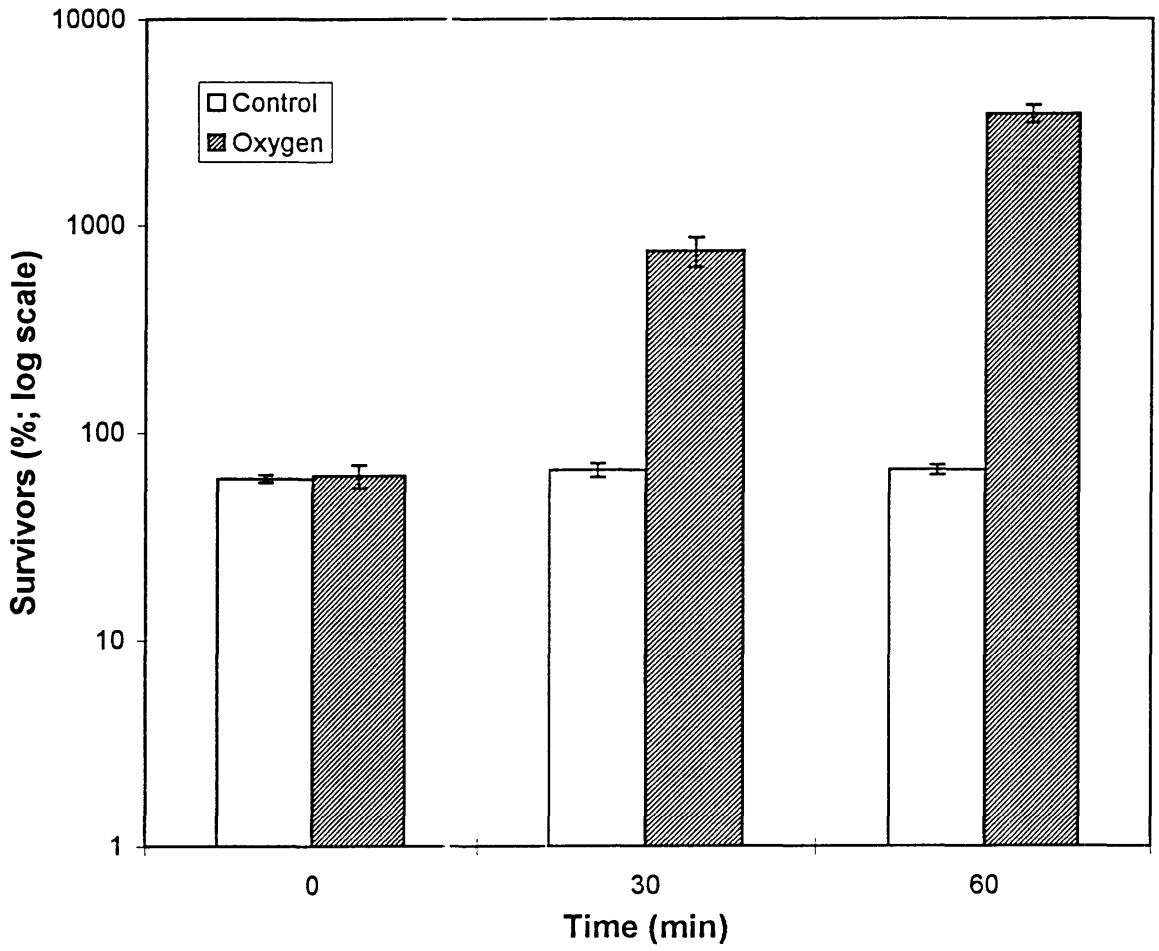

Fig. 4.7 Effect of oxygenation following heat treatment. Cells grown aerobically at 25°C were heat shocked at 37°C for 45 min, followed by a heat stress at 48°C for 30 min. Cells were cooled to 25°C and oxygenated with high purity oxygen for 30 min and 60 min before plating out on 2% glucose YEP-media. Results are the average of three separate experiments.

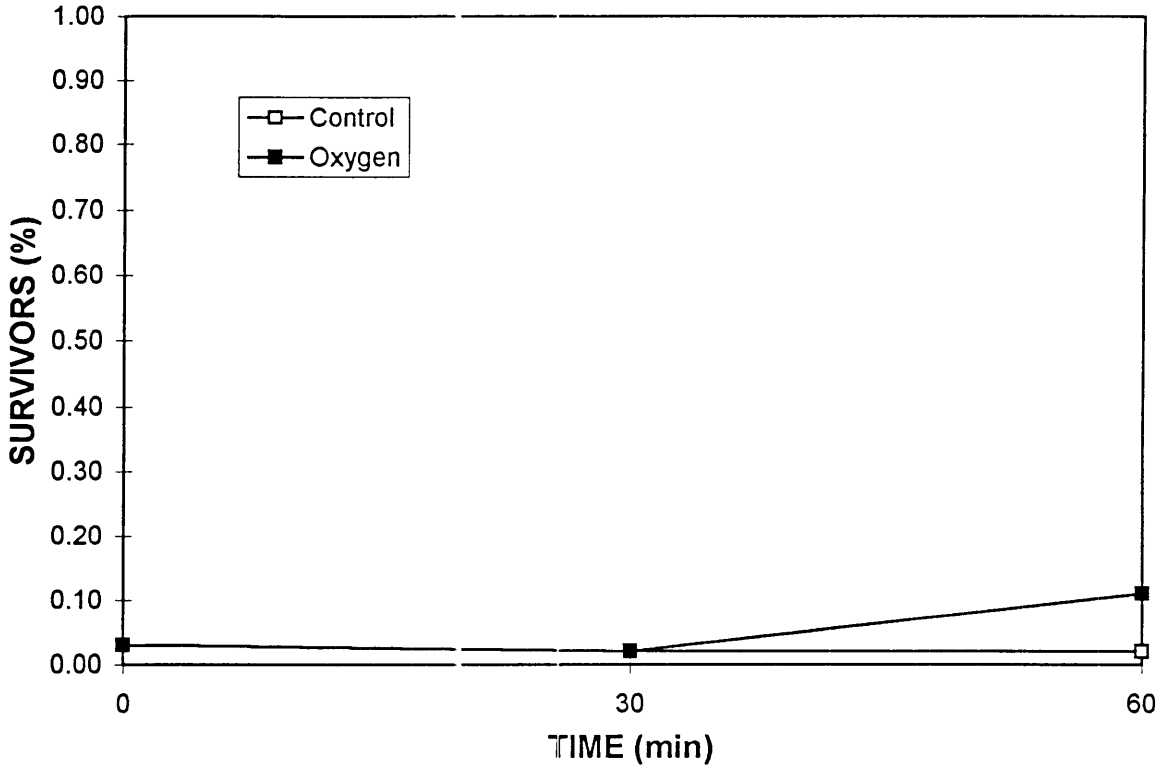

Fig. 4.8 Effect of oxygenation following heat stress. Cells grown aerobically at 25°C were heat stressed at 48°C for 30 min. Cells were cooled to 25°C and oxygenated with high purity oxygen for 30 min and 60 min before plating out on 2% glucose YEP-media. Result from a typical experiment.

1.6. Percentage of budded cells

The percentage of budding cells during oxygenation was determined by examination of cells under the microscope using a haemocytometer. The results are presented in Fig. 4.9 which shows percentage of budded cells during oxygenation at 25°C, and Fig. 4.10 which shows the percentage budding during oxygenation of cells following recovery from heat stress. There was little change in percentage budding during aeration in either case. About 35-45% cells were budding before aeration and this level was unchanged after 2 hr. There was a slight decrease in the percentage of budded cells during aeration at 25°C after 60 min.

2. ANAEROBIC

All experiments described in this section, unless otherwise indicated, were conducted on cells grown anaerobically with ergosterol and oleic acid (C18:1) as lipid supplements.

2.1. Aeration with oxygen during heat treatment

The experimental protocol was similar to that adopted for cells grown aerobically (section 2.9). In a typical experiment, anaerobic cells at around mid-exponential growth phase corresponding to approximately $1x10^7$ cells/ml (abs. 0.3 at 600 nm) were heat shocked from 25°C to 37°C for 45 min and then heat stressed to 48°C for various times. Throughout the heat treatments, cultures were aerated with a constant stream of high purity oxygen. Temperatures were altered by connecting the water-jacketted fermentors, containing 100 ml cell culture in a 250 ml vessel, to a thermocirculator set at the appropriate temperature (37°C or 48°C).

Results for heat treatment of oxygenated anaerobic cells are presented in Fig. 4.11. Survival rates were approximately 4-fold higher in oxygenated cells as compared to non-oxygenated cells. Cell survival decreased with increasing incubation time at 48°C, from 15 min through to 30, 60 and 120 min. However,

the approximate 4-fold difference in % survivors was maintained at the different incubation times. Cells which had been heat stressed directly from 25°C to 48°C for various times during oxygenation showed a lower % survivors compared to the non-oxygenated controls (Fig. 4.12).

2.2. Aeration with oxygen following recovery from heat treatment

Cells were heat shocked and then stressed at 48°C for 30 min. Cells were then allowed to recover at 25°C for 30 min and 60 min, with or without oxygenation, before dilution plate count analysis.

Oxygenation of cells, following heat treatment, for 30 min result in a marked increase, about 7-fold, in cell survival compared to non-oxygenated controls. The survival rate was further increased, about 15-fold following 60 min oxygenation (Fig. 4.13).

2.3. Aeration of oxygen during oxidative stress

Cells were challenged with H_2O_2 (5 mM) and incubated at 25°C over a time course, with or without oxygenation. Result are presented in Fig. 4.14. In the first 15 min period, oxygenation resulted in a 3-fold increase in % survivors. However, at 30 and 60 min exposure, the % survivors in non-oxygenated controls were greater than in the oxygenated samples.

3. TREHALOSE

The trehalose concentration of aerobic and anaerobic cells was determined under various experimental conditions. In the case of aerobic cells, two series of experiments were conducted. One on measurement of trehalose concentration in cells which had been washed once in YEP-media (minus glucose) and the second on unwashed cells, that is cells in the original growth media which generally contained about 0.5% residual glucose. Both cell types were then subjected to a mild heat shock (37°C for 45 min), with or without oxygenation. The results for these experiments are

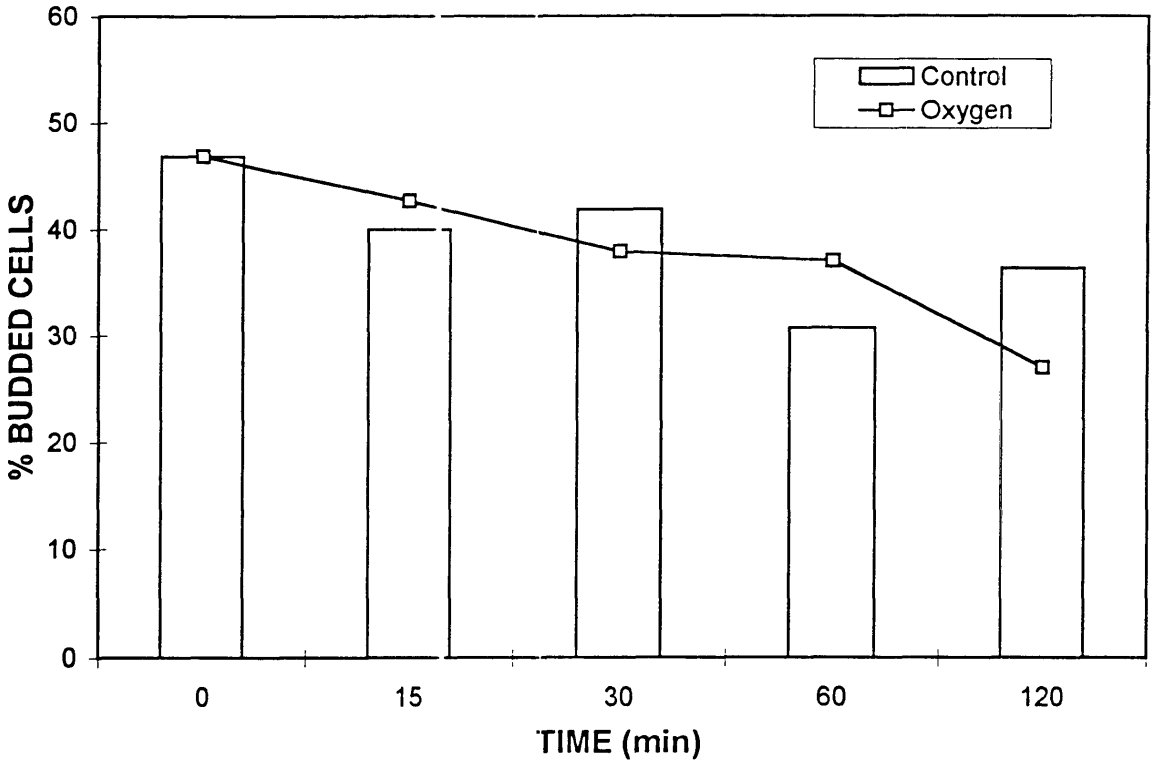

Fig. 4.9 Percentage of budded cells during oxygenation. Cells grown aerobically at 25°C to mid-log phase were aerated with oxygen at 25°C over a 120 min time course.

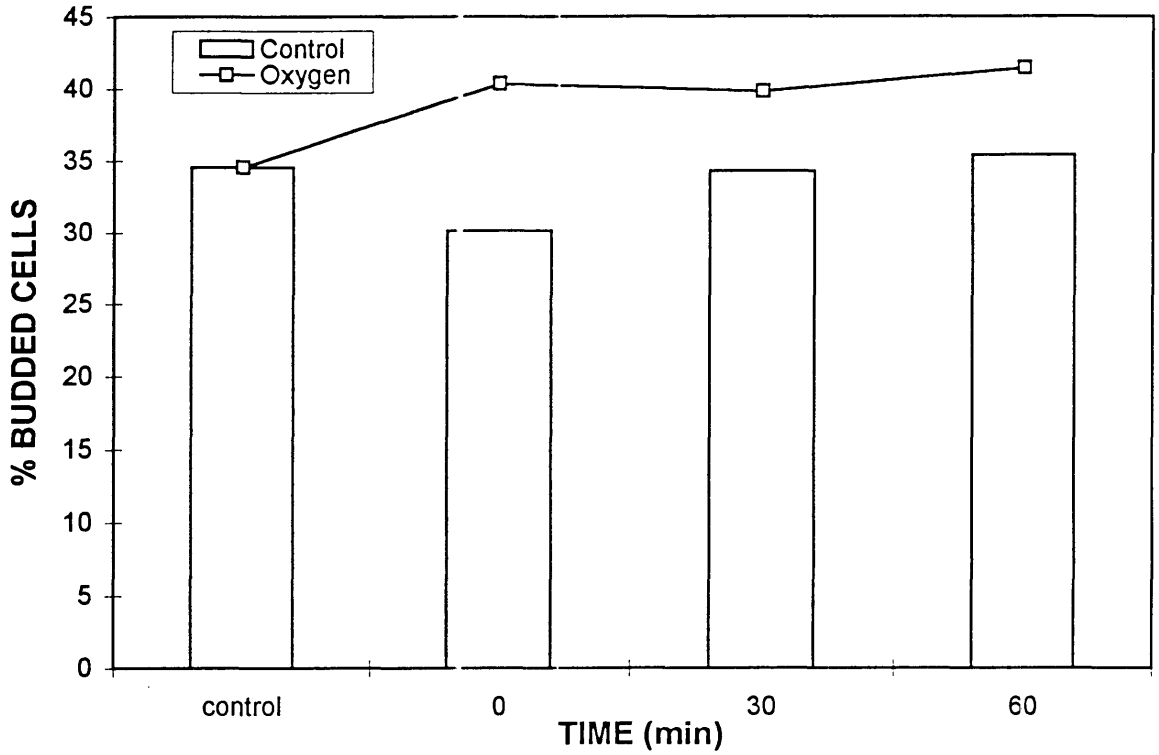

Fig. 4.10 Percentage of budded cells following heat treatment (37°C, 45 min followed by 48°C, 30 min) and oxygenation. Cells were grown aerobically at 25°C, heat shocked/heat stressed and then aerated with oxygen at 25°C over a 60 min time course.

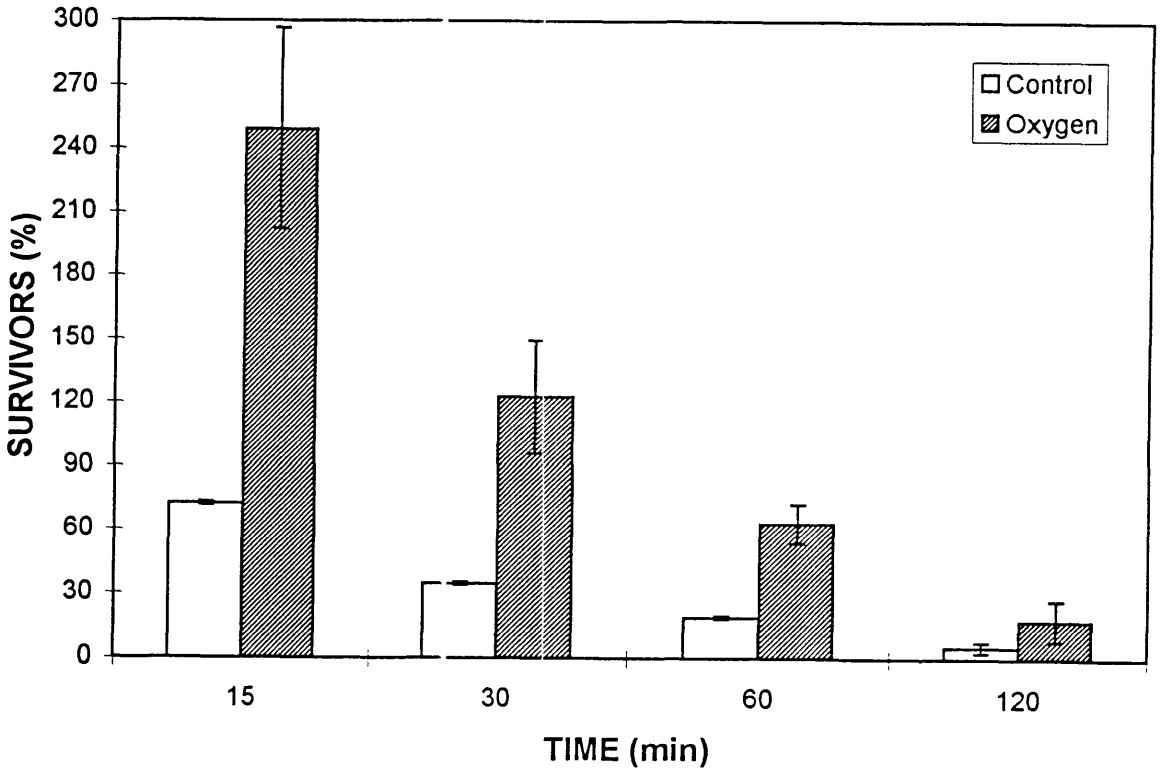

Fig. 4.11 Effect of aeration with oxygen on induced thermotolerance of anaerobically grown cells. Cells were heat shocked to 37°C for 45 min followed by a heat stress at 48°C over a time course, with or without oxygenation Results are the average of three separate experiments.

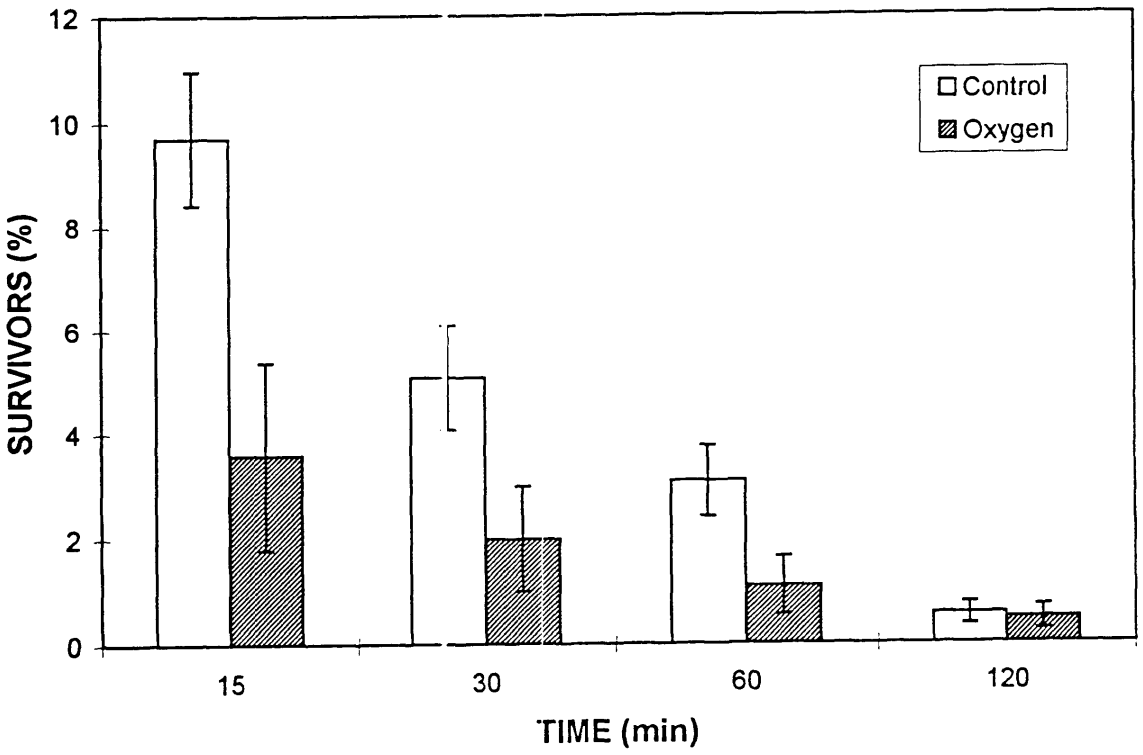

Fig. 4.12 Effect of aeration with oxygen on intrinsic thermotolerance of anaerobically grown cells following heat stress at 48°C over a time course. Results presented are the average of three separate experiments.

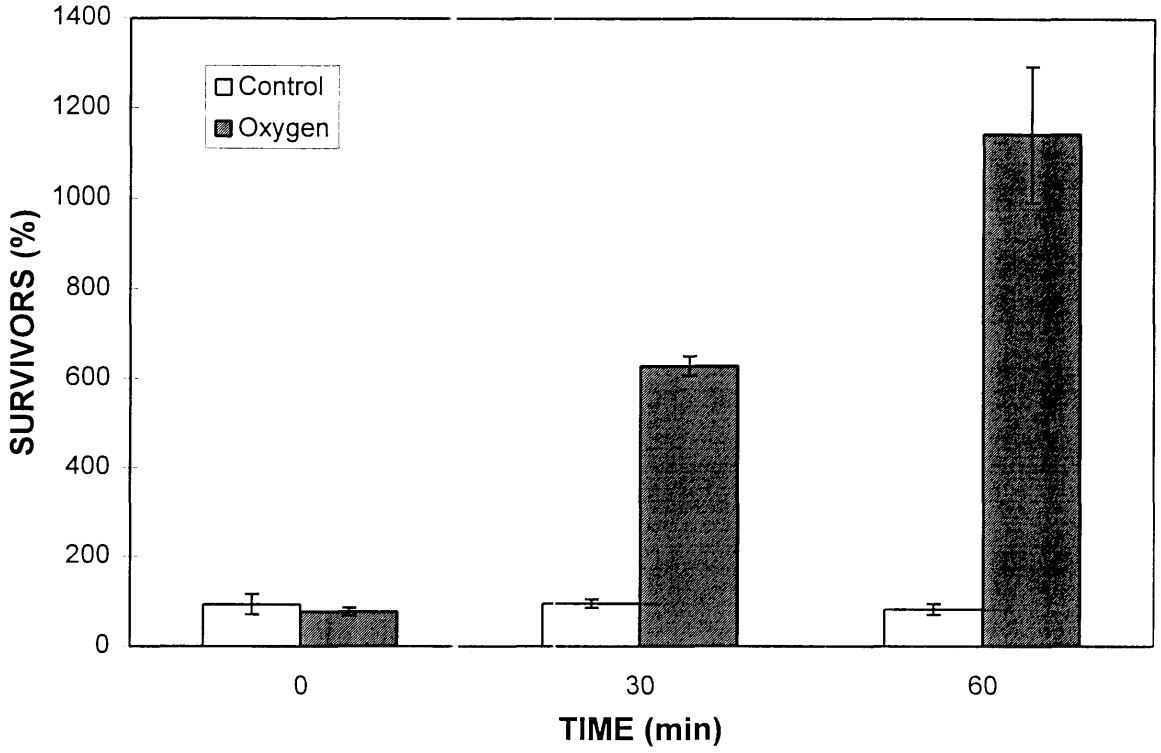

Fig. 4.13 Effect of aeration with oxygen on induced thermotolerance of anaerobically grown cells. Cells were heat shocked at 37°C for 45 min, followed by heat stress at 48°C over a 30 min. Cells were then allowed to recover at 25°C for 30 min or 60 min with or without oxygenation. Results presented are the average of three separate experiments.

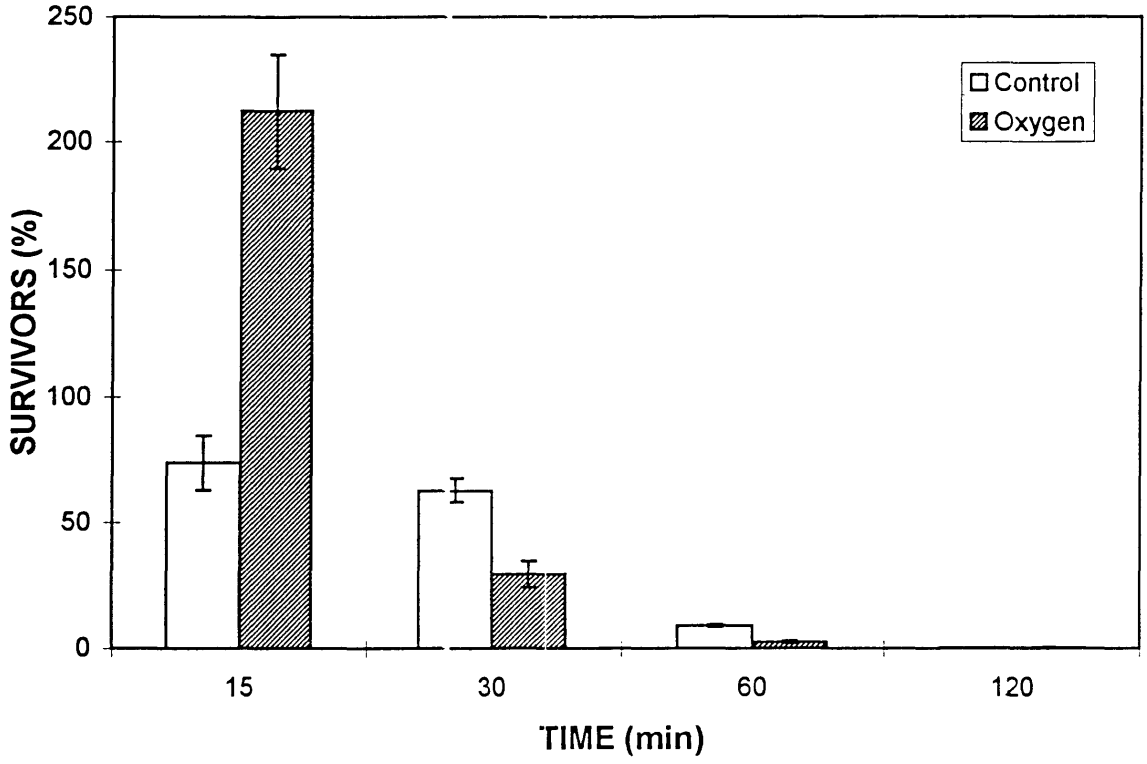

Fig. 4.14 Effect of aeration with oxygen on induced thermotolerance of anaerobically grown cells during oxidative stress. Cells were challenged at 25°C with H_2O_2 (5 mM) over a time course, with or without oxygenation. Results presented are the average of three separate experiments.

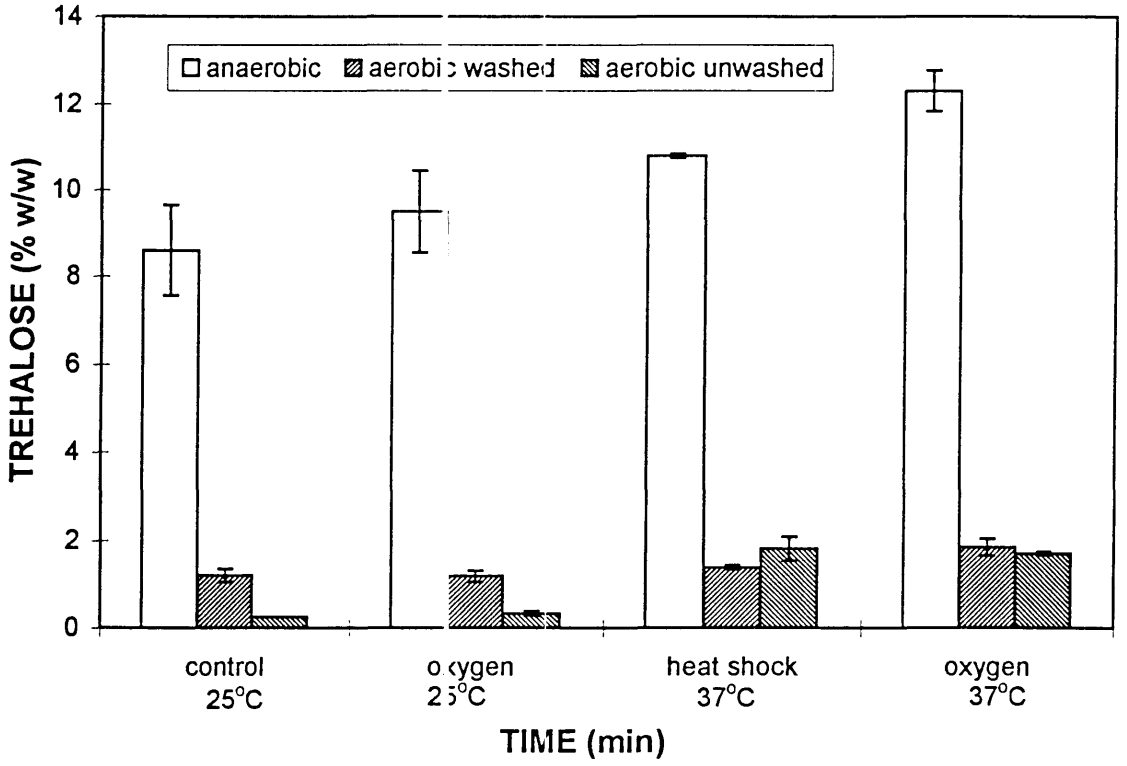

Fig. 4.15 Trehalose content of cells grown aerobically or anaerobically. Anaerobically grown cells were washed and incubated in fresh YEP without glucose. Aerobic washed refers to cells washed in YEP and incubated in fresh YEP media without glucose. Aerobic unwashed refers to cells maintained in their original growth media and then heat shocked to 37°C for 45 min, with or without oxygenation.

shown in Fig. 4.15. Washed aerobic cells showed little change in trehalose content, on average about 1.4% w/w, on oxygenation (for 45 min) at 25°C or on heat shock at 37°C for 45 min. There was a moderate increase (just under 2% w/w) on oxygenation at 37°C for 45 min.

The trehalose concentration of aerobic cells estimated directly from their original growth medium (unwashed cells) was significantly lower (on average approximately 0.3% w/w) than in washed cells (average approximately 1.4% w/w). Oxygenation for 45 min at 25°C did not greatly alter the trehalose concentration of the cells. On the other hand, a mild heat at 37°C for 45 min, with or without oxygenation, induced the synthesis of trehalose, up to about 1.8% w/w.

Anaerobically grown cells had remarkably high concentrations of trehalose, approaching 9% w/w. Oxygenation at 25°C led to a moderate increase in trehalose. A mild heat shock induced the synthesis of trehalose to around 11% w/w. This value increased to over 12% in cells heat shocked in the presence of oxygen.

4. HEAT SHOCK PROTEIN SYNTHESIS

4.1 Aerobic cells

In this study, the synthesis of newly synthesised proteins in cells subjected to heat shock, with or without oxygenation, was examined by measurement of the incorporation of ^{35}S-methionine into protein. A summary of the results for cells grown aerobically at 25°C is presented in Fig. 4.16. A mild heat shock at 37°C for 45 min induced the synthesis of the classical heat shock proteins. These included bands corresponding to hsp 104 (at about 100 kDa), hsp 70 (at 65-78 kDa) and hsp 60 (61 kDa). In addition, bands were also observed around the 35 to 44 kDa region and also at around 30 kDa. There were no obvious additional bands when cells were heat shocked in the presence of oxygen. However, when cells were oxygenated at 25°C for 45 min

additional bands were observed at approximately 40 kDa and 36 kDa. It was noteworthy that the synthesis of some proteins decreased on a heat shock. These proteins were located at around 51 kDa and in the region of 36 to 40 kDa.

4.2. Anaerobic cells

Cells grown anaerobically, in the presence of ergosterol and oleic acid (C18:1) as lipid supplements, were examined for heat and oxygen induced protein synthesis. A mild heat shock induced similar protein bands as that observed with aerobic cells (Fig 4.17). Proteins of apparent molecular mass at about 90, 70, 60, 40 and 30 kDa were observed. On the other hand, no obvious additional protein bands were observed in cells subjected to oxygenation either at 25°C or during heat shock at 37°C.

5. FATTY ACID COMPOSITION

The fatty acid composition of the phospholipid fraction from aerobic cells grown at 25°C was typical of that for *Saccharomyces* yeast (Table 4.2). The main fatty acyl components were palmitoleic acid (C16:1) at around 50% and oleic acid (C18:1) at about 25%. The remaining fatty acyl components were largely saturated fatty acids of the C10:0, C12:0, C14:0 and C16:0 classes. There were moderate changes in fatty acyl composition following temperature upshift, with or without oxygenation. The changes were mainly reflected in an increase in percentage composition of C16:1 and C18:1 with a corresponding decrease in the saturated fatty acids, notably in C16:0.

The fatty acyl composition of cells grown anaerobically with oleic acid (C18:1) or linolenic acid (C18:3) in the growth media was enriched in the corresponding lipid supplement. In the case of C18:1 supplemented cells, the fatty acyl composition was predominantly C18:1 ,approaching 80%, with <5% as C16:1 and the remainder as saturated fatty acids. Cells grown with C18:3 supplement were less enriched (40%) in the lipid supplement and contained a

substantial proportion (about 23%) of C18:1 fatty acid as well as C16:0 (about 25%).

Cells grown aerobically at 25°C consisted of about 50 % C 16:1 and about 25 % C 18:1 (Table 4.2). Changing the temperature of the cells (by shock and stress) for 2 hr as well as addition of oxygen during heat treatment seemed to have little effect on the percentage of C:18 fatty acids. The percentage of C:16 after shock/stress for 2 hr decreased less than 2%, and a shorter time stress (15 min) produced a decrease of about 4%. There were similar changes in the percentage of this fatty acid after aeration with oxygen during stress.

In contrast to aerobically grown cells, temperature and oxygen induced changes were evident in the percentage of the unsaturated fatty acids C16:1 and C18:1 in anaerobic cells supplemented with C18:1. Increasing temperature (by heat shock/stress) resulted in increase percentage of C16:1 and a decrease in C18:1. Oxygenation during heat treatment lead to similar changes in fatty acid composition. It was noteworthy that there was a substantial increase in C18 following oxygenation.

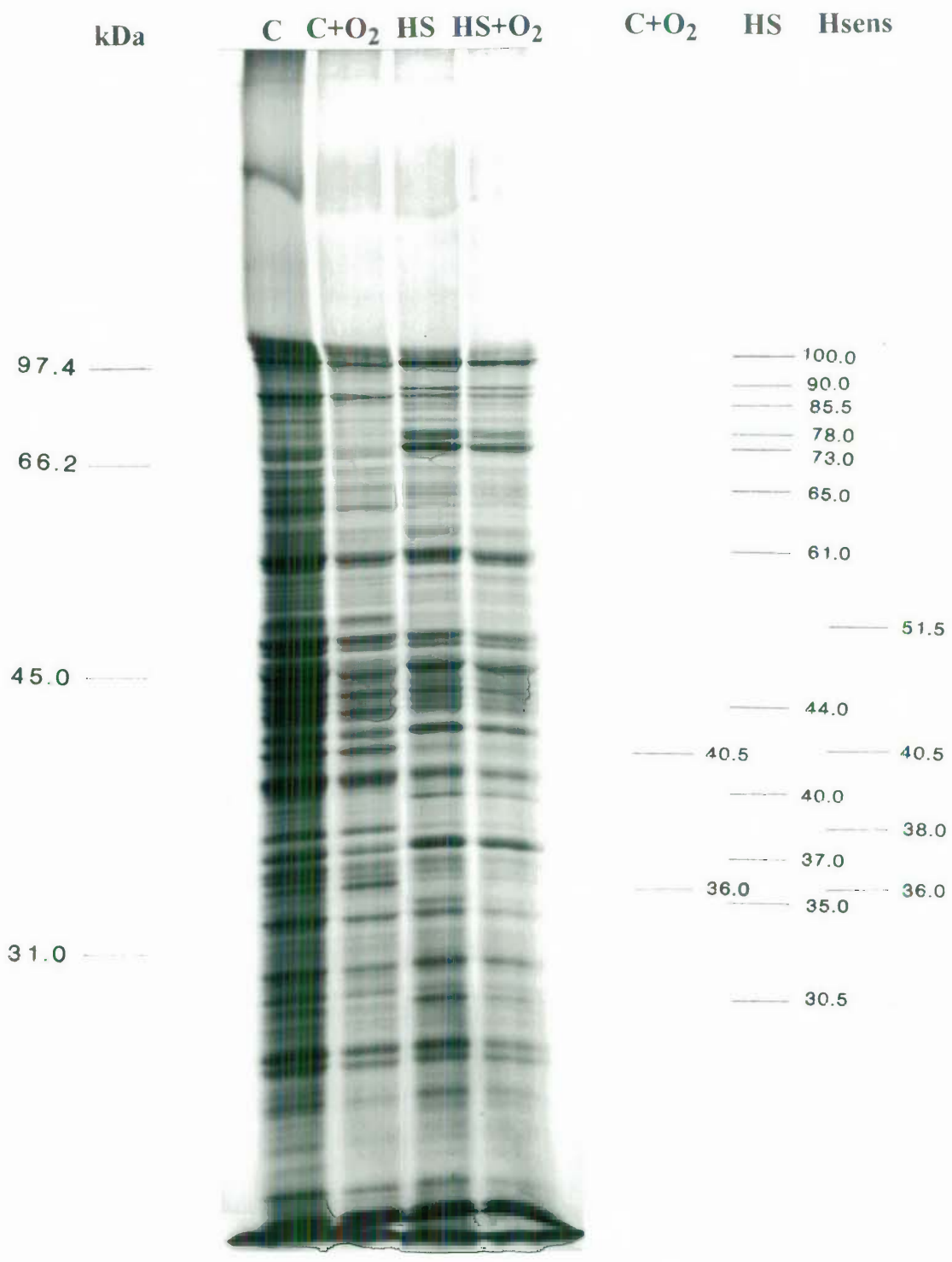

Fig. 4. 16. Autoradiogram of protein synthesis of cells at 25°C or exposed to heat shock (37°C) for 45 min with or without oxygenation. Cells grown aerobically in 2% glucose YEP were washed and resuspended in 2% glucose YNB. Cells were labelled as indicated in Methods. C, C+O_2, HS, HS+O_2 and Hsens refer to control at 25°C, control + oxygenation at 25°C, heat shock at 37°C, heat shock + oxygenation at 37°C and heat sensitive, respectively. The latter refers to proteins with decreased synthesis on heat shock.

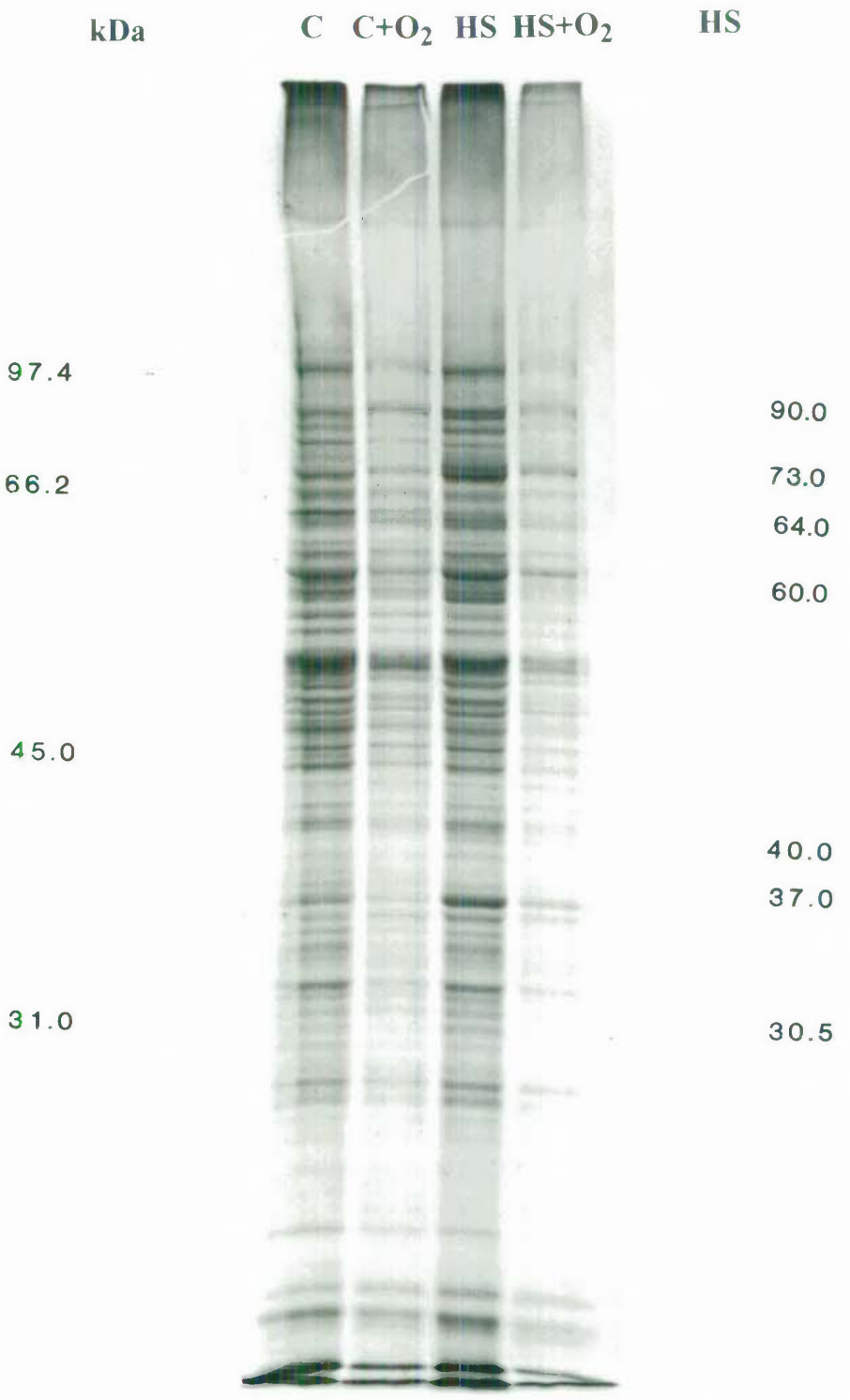

Fig. 4. 17. Autoradiogram of protein synthesis of cells grown anaerobically at 25°C or exposed to heat shock (37°C) for 45 min with or without oxygenation. Cells grown anaerobically in 2% glucose YEP were washed and resuspended in 2% glucose YNB . Cells were exposed to 37°C for 5 min prior to labelling as in Methods. C, C+O_2, HS, HS+O_2 refer to control, control + oxygenation at 25°C, heat shock and heat shock + oxygenation at 37°C, respectively.

Table 4. 2. Fatty acyl composition of aerobic and anaerobic cells subjected to heat stress. Cells at mid-to-late exponential growth phase were subjected to heat shock at 37°C for 45 min and heat stress at 48°C for various times as indicated with or without oxygen (control). Fatty acyl composition was expressed as a percentage of the total fatty acid. Result from a typical experiment.

TREATMENT			Fatty acids								
			C10	C12	C14	C14:1	C16	C16:1	C18	C18:1	C18:3	Unsaturation index*
Anaerobic												
	Oleic acid	Control	-	0.9	2.5	-	14	2.9	1.9	77.5	-	0.80
		Shock/stress (120 min)	-	1.2	2.9	-	16.4	7.4	3.6	68.5	-	0.76
		Ss + oxygen (120 min)	-	0.5	1.5	-	15.7	8.5	6.3	67.5	-	0.76
	Linolenic acid	Control	-	0.9	3.6	-	25.7	2.4	4.6	22.7	40	1.5
Aerobic		Control	1.22	1.5	4	0.9	16.1	48.7	2.5	25	-	0.75
		Shock/Stress (15 min)	0.5	1.3	3	1.6	12.4	51.3	2.6	27.3	-	0.80
		Shock/Stress (120 min)	-	1.5	3.2	1.7	14.5	49.6	2.8	26.8	-	0.78
		SS + oxygen (15 min)	-	0.9	2.7	1.4	12.2	53.3	2.8	26.7	-	0.80
		SS + oxygen (120 min)	0.3	0.7	3	1.3	15.9	50.4	3	25.4	-	0.77

* Unsaturation index = (1 x % monoene + 2 x % diene + 3 x % triene) / 100 (Kates and Baxter, 1962)